Characterisation of the KA1 & KA2 Domains and Interaction with APC/C.

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Declaration

I declare that:

a. This thesis was composed by myself

b. The research presented is my own unless otherwise stated

c. This work has not been submitted for any other degree for any other degree or professional qualification.

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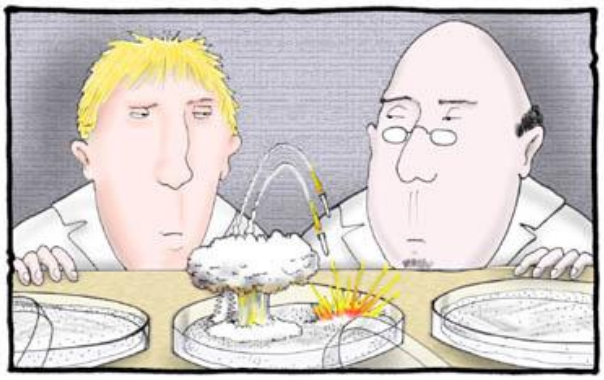
2011

Y

Yeast, n.

Gregarious single-celled organism whose first evolutionary priority was to develop the capacity to synthesize alcohol. Usually well behaved, but can become rowdy in groups.



Once again, war breaks out in the middle yeast.

i. Acknowledgements

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ii. Abstract

Ubiquitin is a highly conserved 76 amino acid protein which is a unique and versatile signalling molecule. Ubiquitin can be attached by an isopeptide bond between its C-terminal diglycine to a lysine residue of a target substrate. However, it can also bind to itself though one of its own seven lysine residues allowing the formation of different chain types. These chains act as signals for different pathways, such as DNA damage repair, and in particular lysine-48 chains signal for proteins to be degraded via the proteasome by the ubiquitin proteasome system (UPS). This allows cells to control the concentration of proteins which is important in triggering cellular events, such as cyclin levels in cell division. Whilst old and incorrect proteins need to be removed so they do not interfere with normal processes.

In order to recognise and ubiquitinate substrates an enzyme cascade has evolved. Ubiquitin is transferred from an ubiquitin activation enzyme (E1) to an ubiquitin conjugating enzyme (E2). The E2 which along with a ubiquitin ligase (E3) ubiquitinates a specific substrate. Research has focused on the E3 ligases since they are responsible for identifying substrates. One important ligase is the anaphase promoting complex/cyclosome (APC/C) which is responsible for faithful segregation of chromosome during mitosis. Failure to regulate this process can lead to aneuploidy, one of the main causes of cancer. It is therefore important to understand the function and regulation of APC/C and the UPS.

This work firstly shows that four *S. pombe* kinases, Ssp2, Ppk9, Kin1 and Chk1 all contain a kinase associated 1 (KA1) or KA2 domain which they use to interact specifically with APC/C when it contained an unphosphorylated form of a subunit called Cut9. Yeast two hybrid and native far Westerns demonstrated that the KA domains interact with the APC/C co activator Slp1. Phosphorylation assays showed that three of these kinases phosphorylated a ~30kDa band of the APC/C complex which was shown to be Mad2, an important subunit of the APC/C inhibitor complex the mitotic checkpoint complex (MCC). These finding suggest a new role for KA contain kinases as regulators

of APC/C activity. Future studies to identify the residues of Mad2 which are phosphorylated by these kinases, as well as the binding site of Slp1 that the KA domains recognise, would provide a more detailed understanding of the molecular mechanisms involved in regulating APC/C activity.

Secondly, this study investigated the role of the ubiquitin associated (UBA) domains in the *S. pombe* shuttle factor Rhp23. This protein can recognise the proteasome via an ubiquitin like (UBL) domain and ubiquitin chains via one of two UBA domains: an internal UBA1 and a C-terminal UBA2. To dissect the different functions of these two UBA domains point mutations were made that abolished the domains ability to recognise ubiquitin without altering the protein structure. The minimal domains and full length domains were tested *in vitro* and *in vivo*. These surprising results showed that the domains act differently in isolation when compared to the full length protein. They also demonstrate that the UBA1 domain is responsible for ubiquitin recognition in Rhp23, whilst the UBA2 domain appears to have little to no binding ability.

iii. Abbreviations

A/Ala	alanine
AAA	ATPase associated with various cellular activities
ADP	5' adenosine diphosphate
AID	auto-inhibitory domain
AMP	5' adenosine monophosphate
AMPK	5' adenosine monophosphate-activated protein kinase
APC/C	anaphase promoting complex/cyclosome
APS	ammonium persulfate
ASC	associated with SNF1 kinase complex
ATM	ataxia-telangiectasia-mutated
ATP	5' adenosine triphosphate
ATR	ataxia telangiectasia and Rad3-related
BSA	bovine serum albumin
Bub	budding uninhibited by benimidazole
CBS	cystathionine-beta-synthase
Cdc	cell division cycle
Cdk	cyclin dependent kinase
Cds	checkpoint DNA synthsis
CKI	cyclin dependent kinase inhibitor
cs	cold sensitive
C-terminal	carboxyl-terminal
CUE domain	coupling of ubiquitin conjugation of endoplasmic reticulum degradation domain

cut	cell untimely torn
Cys	cysteine
D-box	destruction box
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DUB	deubiquitinating enzyme
E1	ubiquitin activating enzyme
E2	ubiquitin conjugating enzyme
E3	ubiquitin ligase
E. coli	*Escherichia coli*
EDTA	ethylenediamine tetra-acetic acid disodium salt
EMM	Edinburgh minimal medium
ERAD	endoplasmic reticulum associated protein degradation
F/Phe	phenylalanine
G_1	growth/gap phase 1
G_2	growth/gap phase 2
G418	geneticin
GAT domain	GGA and Tom1 domain
GBD	glycogen-binding domain
GFP	green fluorescent protein
Gly	glycine
GLUE domain	GRAM-like ubiquitin binding in Eap45 domain

GST	glutathione transferase
H/His	histidine
HA	haemaglutinin
HECT	homologous to E6-AP carboxyl terminus
HRP	horseradish peroxidise
HU	hydroxyurea
HYG	hygromycin B
IPTG	isopropylthio-β-D-1-thiogalactopyranoside
IR	isoleucine-arginine dipeptide
KA1/2	kinase associated 1/2 domain
kb	kilobase pairs
kD	kilodaltons
KIS	kinase interacting sequence
L/Leu	leucine
LB	Lysogeny broth
Lys	lysine
M	mitotic phase
M/Met	methionine
Mad	mitotic arrest deficiency
MAP	microtubule-associated protein
MARK	microtubule affinity regulating kinases

MCC	mitotic checkpoint complex
ME	malt extract
Met	methionine
$MgCl_2$	magnesium chloride
min	minutes
MMS	methyl methanesulfonate
MPF	M phase factor
mts	MBC-resistant temperature sensitive
NAT	nourseothricin
Nedd8	neural precursor cell expressed and developmentally down-regulated 8
NER	nucleotide excision repair
nmt	no message thiamine
N-terminal	amino-terminal
NZF domain	Npl4 zinc finger domain
OD	optical density
oligo	oligonucleotide
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PAZ domain	polyubiquitin-associated zinc binding domain
PBS	phosphate buffered saline
PBST	PBS tween
PCR	polymerase chain reaction

PEG	polyethylene glycol
PEST	rich in proline, glutamic acid, serine and threonine
PHD	Pleckstrin-homology domain
Phe	phenylalanine
PKA	protein kinase A
Plk	polo like kinase
PMG	pombe glutamate media
Ppk	putative protein kinase
Pru domain	pleckstrin-like receptor for ubiquitin domain
Ptr	poly(A)+ RNA transport
Rhp	rad homolog pombe
RING	really interesting new gene
rpm	revolutions per minute
Rpn	regulatory particle nonATPase
Rpt	regulatory particle ATPase
rt	room temperature
S	synthesis phase
SAC	spindle assembly checkpoint
SC	synthetic complete media
S. cerevisiae	*Saccharomyces cerevisiae*
SCF	SKP1–CUL1–F-box-protein
SDS	sodium dodecyl sulphate
SE	standard error
Ser	serine

sec	seconds
Snf	sucrose non-fermenting
S. pombe	*Schizosaccharomyces pombe*
Ssp	suppressor of sts5 and ppe1 mutants
SUMO	small ubiquitin-like modifier
TAE	Tris base, acetic acid and EDTA buffer
TAP	tandem affinity purification
TBS	Tris buffered saline
TBZ	thiabendazole
TE	Tris EDTA buffer
TEMED	tetramethylethylenediamine
Thr	threonine
TPR	trichotetrapeptide repeats
tRNA	transfer ribonucleic acid
Tyr	tyrosine
ts	temperature sensitive
Ub	ubiquitin
UBA domain	ubiquitin-associated domain
UBD	ubiquitin binding domains
U-box domain	UFD2-homology domain
Ubc	ubiquitin conjugating enzyme
UBL protein	ubiquitin like protein
Ubl domain	ubiquitin like domain
UEV domain	ubiquitin E2 variant domain

UIM	ubiquitin interaction motif
U/Ura	uracil
UPS	ubiquitin proteasome system
UV	ultraviolet
vWA domain	von Willebrand factor type A domain
VHS domain	Vps-27, HRS and STAM domain
W/Trp	tryptophan
WPW	Wolff-Parkinson-White
YES	yeast extract supplemented media
YPAD	yeast extract-peptone-adenine-dextrose media

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Chapter1
Introduction

1.1 *S. pombe*

Schizosaccharomyces pombe, commonly called fission yeast, is a single celled free living organism that is a member of the *Schizosaccharomycetaceae* family of fungi. It shares some similarities to another yeast, *Saccharomyces cerevisiae*, both of which are commonly used as a model organisms for research. Both these yeasts have been extensively used as genetic models as their genomes, which are fully sequenced, are easy to manipulate. As they exist as haploids, they usually have one copy of each gene. Another advantage that arises with these well studied organisms is that there are now many reagents and protocols available (Forsburg & Rhind 2006). *S. pombe* has a genome of 13.8Mb which consists of three chromosomes as well as a 20kb mitochondrial genome. Together the genome encodes up to 4940 Open Reading Frames (ORF). Many of these genes have homologues in higher organisms (Wood et al. 2002).

However despite those similarities there are many differences between the two yeasts which were estimated to have separated around 1000 million years ago. Under normal conditions *S. pombe* exist as haploid whilst *S. cerevisiae* are diploid, although lab strains can be manipulated to produce diploids or haploids respectively. Sequencing of their genomes revealed that *S. cerevisiae* has undergone a large scale duplication, something *S. pombe* has not done. *S. pombe* cells are 12–15µm in length and therefore larger than *S. cerevisiae* 5–10µm which makes them easier to observe. Also *S. pombe* undergoes medial fission division similar to mammalian cells whilst *S. cerevisiae* divides by forming a bud, giving rise to its common name of budding yeast (Wood et al. 2002; Forsburg & Rhind 2006).

S. pombe was the model organism used by Paul Nurse and colleagues to dissect the components of the cell cycle. One of the features of fission yeast that allowed them to do this is that during the cell cycle fission yeast increases in length. By simply measuring the cell you can determine what stage it is in. This work lead to Sir Paul Nurse, Leland

Hartwell and Richard “Tim” Hunt being awarded the Nobel Prize in Physiology or Medicine in 2001 *"for their discoveries of key regulators of the cell cycle"* (Nobelprize.org 2011).

1.2 Cell Cycle

In order to grow, repair, develop and reproduce all cells must replicate. Cells undergo a tightly controlled cell cycle which allows them to divide and pass on their DNA and cellular components to a new sister cell. The cell cycle is divided into two stages, interphase and mitosis. Interphase is the longest stage which consists of G_1, S and G_2. G_1 is a gap phase in which cells can either commit to divide and prepare to synthesize DNA or enter G_0, a state in which cells are no longer in the cell cycle. DNA is synthesis in S phase causing the cell to temporarily become polyploid. The final stage of interphase is G_2 in which cells prepare to enter mitosis. Mitosis is sub divided into prophase, metaphase, anaphase and telophase. Prophase is defined by the condensing of chromosomes, which attach to spindle microtubules via the kinetochore before metaphase, when bound chromosomes align at the metaphase plate. The next stage is anaphase, in which attached chromosomes separate into sister chromatids via the degradation of the cohesion complex which holds them together. The microtubules then pull the sister chromatids to opposite poles of the cells to form two sister nuclei. Finally during telophase the two new nuclei are formed and the cell undergoes cytokinesis to form two cells (Lodish et al. 2004).

1.2.1 Cdks & Cyclins

The whole cell cycle is controlled by Cyclin Dependent Kinases (Cdk) and cyclins. Cdks are serine threonine kinases which bind to certain cyclins. Cyclins recognise specific target proteins and allows the Cdk to phosphorylate them. The levels of Cdks in the cell remains constant throughout the cell cycle but their activity is regulated by binding cyclins, Cyclin dependent Kinase Inhibitor (CKI), phosphorylation and controlling their location within the cell. However, in mammals the concentration of cyclins varies since

they contain Destruction-boxes (D- boxes) or PEST sequences (rich in proline (P), glutamic acid (E), serine (S) and threonine (T) residues) which marks them as targets for ubiquitination and degradation when they are no longer required. In mammalian systems Cdk6 and Cdk4 bind to D type Cyclin during G_1. At the junction between G_1 and S phase Cdk2 binds to CyclinE, then during S phase Cdk2 binds CyclinA. The transition between G_2 to mitosis is regulated by Cdk1 and CyclinA whilst Cdk1-CyclinB is active during mitosis (reviewed in Schafer 1998; Vermeulen et al. 2003).

S. pombe differs from mammals in that it only possesses one essential Cdk called Cell Division Cycle 2 (Cdc2). It also expressed one essential mitotic cyclin, called Cell Division Cycle 13 (Cdc13) and three non essential cyclins called Cig1, Cig2, Puc1. Instead of using different combinations of Cdks and cyclins, fission yeast uses the concentration of Cdc2-Cdc13, also known as the M Phase Factor (MPF), to control cell division. At G_1 the concentration of Cdc2-Cdc13 is very low, then at S phase the concentration rises to an intermediate level. Mitosis is signalled by a high concentration of Cdc2-Cdc13. When Cdc2-Cdc13 is formed it moves into the nucleus but is inactivated by the Wee1 protein kinase which phosphorylates Cdc2. This phosphorylation is removed by the phosphatase Cdc25 at G_2 activating the MPF. Positive feedback loops are set up since Wee1 is inactivated by MPF whilst Cdc25 is activated by it. This system causes a sharp peak of Cdc2-Cdc13 activation at the transition of G_2-M phase. In order to exit mitosis, the MPF needs to be inactivated by removal of Cdc13, which is carried out by APC/C^{Slp1} and APC/C^{Ste9} which recognises and ubiquitinates Cdc13 causing it to be degraded. Cdc13 is continuously translated and as a result Cdc2-Cdc13 starts to reform at G1. To repress its activity until it is needed, the CKI Rum1 binds to the Cdc2-Cdc13 dimer. However, when the cell increases in size Cdc2 has enough activity to phosphorylate Ste9 causing it to dissociate from APC/C and also phosphorylate Rum1 removing it from the MPF complex. This allows the cell to progress to S phase and the cycle starts again (Lodish et al. 2004; Sveiczer et al. 2004).

1.2.2 Checkpoints

In order to ensure that the correct amount of good quality undamaged DNA is passed on to each cell, the cycle contains several checkpoints, which serve to detect any problems that may occur at different phases and halt the cell cycle. This gives the cell time to repair the problem before continuing to the next stage of the cycle, or if the problem cannot be rectified the cell can undergo apoptosis. The term "checkpoint" was first defined by Hartwell and Weinert when they discovery that irradiation of WT budding yeast caused the cells to undergo a cell cycle delay after G_2, whilst *rad9* mutants could carry on dividing normally, even though their DNA was damaged, which eventually led to lethality (Weinert & Hartwell 1988; Hartwell & Weinert, 1989).

Since this important finding several checkpoints have been found in the cell cycle including: four DNA damage checkpoints at i) G_1 phase ii) G_1-S phase boundary iii) S phase iv) G_2-M phase boundary as well as v) an unreplicated DNA checkpoint at G_2-M phase boundary vi) a spindle assembly checkpoint at metaphase to anaphase boundary (see section 1.6) and vii) a chromosome segregation checkpoint at telophase known as the NoCut pathway in yeast (Forbes & Enoch, 2000; Vermeulen et al. 2003; Lodish et al. 2004; Norden et al. 2006).

In mammalian cells the DNA damage and unreplicated DNA checkpoints are controlled by Ataxia-Telangiectasia-Mutated (ATM) and ATaxia and Rad3 related (ATR). These kinases recognise DNA damage and as a result, phosphorylate p53, causing the transcription of many genes including p21 which is a CKI. p21 inhibits Cdk-cyclin activity causing the cell cycle to be blocked until the damage can be rectified. Alternatively ATM and ATR can block the cell cycle in a p53 independent manner by activating Chk1 and Chk2 (the function of Chk1 will be explained fully in section 1.9.3). These kinases phosphorylate Cdc25 causing it to be sequestered by binding to a 14-3-3 protein so it cannot enter the nucleus, and, therefore, will not remove inhibitory phosphate groups from Cdks. ATR also activates Chk1 before mitosis, as part of the unreplicated DNA checkpoint which stops cells dividing if they have not fully replicated their DNA (Schauber et al. 1998; Vermeulen et al. 2003; Lodish et al. 2004).

The remaining spindle assembly and chromosome segregation checkpoints are controlled through regulating the activity of the enzyme APC/C. These checkpoints ensure that all the chromosomes have attached to spindles before entering anaphase and also monitor chromosomal segregation is completed before exiting mitosis respectively (Vermeulen et al. 2003; Lodish et al. 2004). The role and regulation of APC/C as well the spindle assembly checkpoint will be discussed in sections 1.5 and 1.6.

1.3 Protein Degradation

For a long time the timing of transcription and translation of proteins by cells has been of great interest. The main focus has been put on the importance of proteins being expressed at the right time, but little research has concentrated on the removal of proteins from the cell.

How cells orchestrate the degradation of proteins is vital for the proper function and survival of the cell. Degradation of mis-folded proteins is essential in order that the native proteins may follow their natural function without interference. Order is also maintained by timing the removal of old or surplus protein (Hershko et al. 1982). One of the best examples of this is the controlled progression through the cell cycle by degradation of cyclins; for example cyclinB (Evans et al. 1983; Peters 2006). Where there is inefficient removal of proteins, medical conditions such as neurodegenerative diseases (for example Alzheimer's), can arise due to the formation of protein aggregates (Matsumoto et al. 2004). There are several methods that cells employ to remove unwanted proteins such as autophagy, the Endoplasmic Reticulum Associated Protein Degradation (ERAD) pathway or being directed to the proteasome where they are unfolded and then degraded in an ATP dependent manner as they pass through the organelle.

1.4 Ubiquitination

1.4.1 Structure & Enzymatic Cascade

Ubiquitin is a small 8 kDa protein made up of 76 amino acids which form a distinct tertiary structure known as the β-grasp fold (Figure 1) (Winget & Mayor 2010). Ubiquitin is highly conserved across eukaryotic organisms and is utilised as a signalling molecule as it is able to covalently tag substrate proteins via an enzymatic cascade (Figure 2). A ubiquitin-activating enzyme (E1) uses ATP to adenylate ubiquitin, which causes it to become activated (Hershko et al. 1981). This active form of ubiquitin can bind to the E1 via the sulfhydryl group of one of its cysteine residues forming a thiol ester. The E1 then passes ubiquitin to an ubiquitin-conjugating enzyme (E2). This uses one of its cysteine residues in the catalytic site form a thiol ester with ubiquitin (Hershko et al. 1983). The E2s can then either transfer the ubiquitin to an ubiquitin ligase enzyme (E3), which recognises specific substrates, and then ubiquitinates them or the E2s can work together with an E3 to directly ubiquitinate a substrate without transferring the ubiquitin to the E3. The E3 ligases forms a covalent isopeptide bond between the C-terminal glycine carboxyl group of ubiquitin and the ε-amino group of a lysine from the target protein (Ciechanover et al. 1981; Hershko et al. 1983; Hershko et al. 1986; Nandi et al. 2006). Single or multiple mono ubiquitination can be used to signal processes such as DNA damage repair, histone regulation and endocytosis (Hicke 2001; Hoeller et al. 2006). In all organisms the ubiquitin enzyme cascade forms a hierarchy, usually with a few E1s at the top. In *S. pombe* there is one E1: Poly(A)+ RNA TRansport 3 (Ptr3) (Azad et al. 1997) and several E2s including: UBiquitin-Conjugating enzyme 1 (Ubc1), Ubc4, Ubc6, Ubc7, Ubc8, Ubc11, Ubc13, Ubc14, Ubc15, Ubc16, Rad Homolog Pombe 6 (Rhp6) and Mms2 (C. M. Pickart 2001). As in other organisms, fission yeast encodes many different E3 ligases which all recognise specific target proteins.

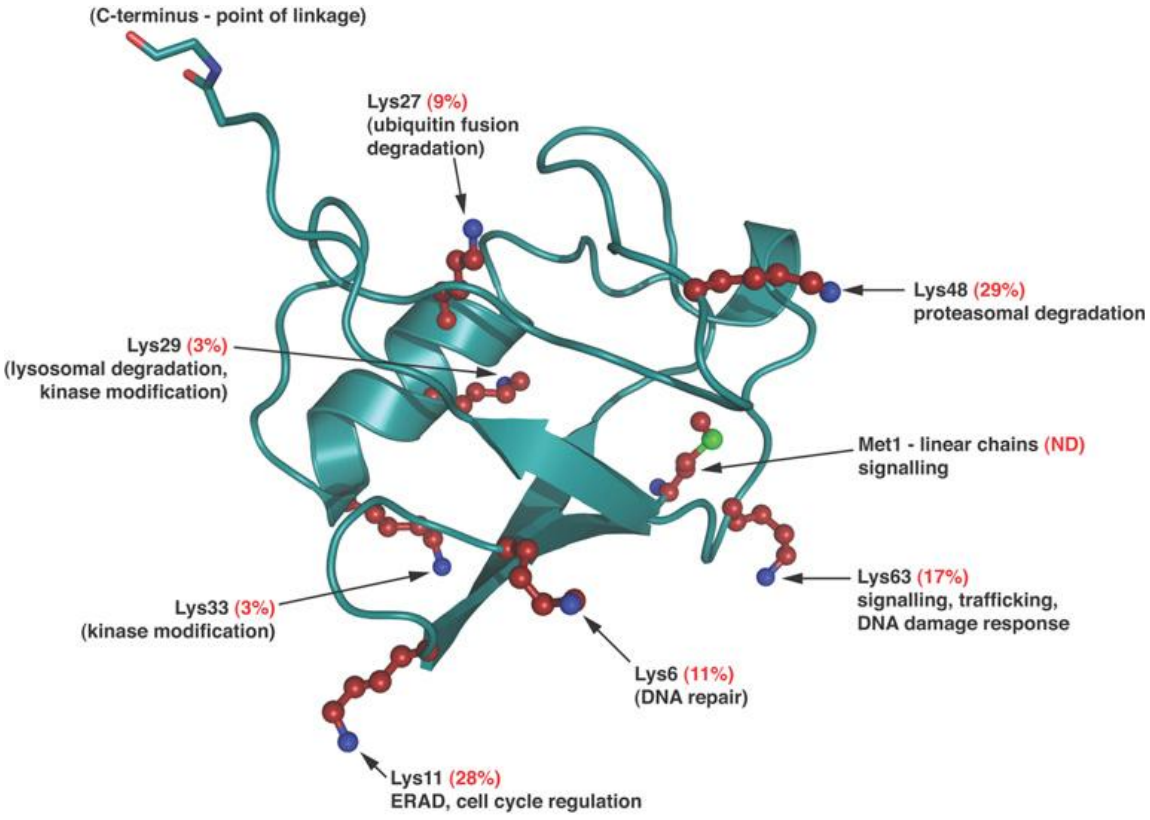


Figure 1: Tertiary Structure of Ubiquitin.

A cartoon of the structure of *S. cerevisiae* ubiquitin. Lysines represented as "ball and stick" cartoon. Percentages shown in red next to lysine residues relate to the relative amount of linkage formed by the lysine residues in *S. cerevisiae* (Komander 2009).

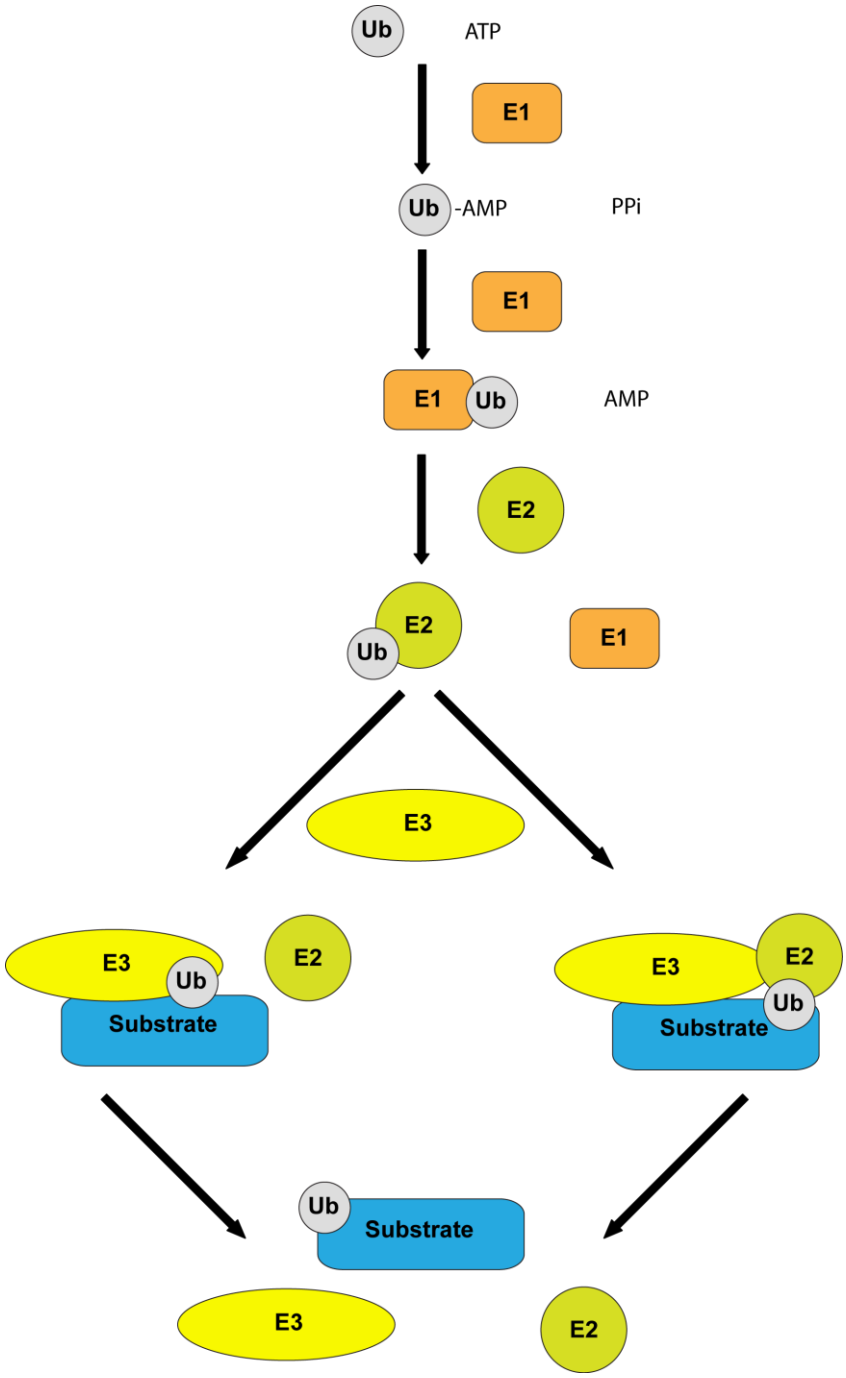


Figure 2: Enzymatic Ubiqutin Cascade.

Ubiquitin is activated by an E1 that utilises ATP to form an adenylate ubiquitin. This form of ubiquitin can then bind to the E1 which passes the ubiquitin moiety onto the E2. The E2 can then either pass the ubiqutin onto an E3 to form a chemical intermediate which can ubiquitinate a substrate at a lysine residue (e.g HECT), or it can work with the E3 as a co-factor to ubiquitinate a substrate (e.g. RING).

1.4.2 Chain Formation

What makes ubiquitin such a diverse signalling molecule is its ability to bind to itself through one of its own 7 lysine residues: K6, K11, K27, K29, K33, K48 and K63 to form chains (Figure 1). These different chains are used as signals for different pathways (Pickart & Fushman 2004). For example, K63 chains can signal for DNA repair and activation of kinases, but K48 chains usually mark proteins for degradation via the proteasome, a process which is known as the Ubiquitin Proteasome System (UPS) (Pickart & Hofmann 1999; Thrower et al. 2000). A fourth enzyme (E4) has been described which specifically recognises mono-ubiquitinated targets and facilitates poly-ubiquitin chain formation (Koegl et al. 1999).

1.4.3 Removing Ubiquitin

Ubiquitination is a dynamic signal as the modification can also be removed from proteins by proteases called DeUBiquitin enzymes (DUB), in fission yeast known as Pad1, Uch2 and Ubp6 (O'Donoghue & Gordon 2008). In *S. pombe*, there are four genes which encode ubiquitin bound to a ribosomal protein. There is also a fifth gene that encodes a chain of four ubiquitin moieties fused together, which is expressed in times of cellular stress, when protein degradation is up-regulated. Ubiquitin needs to processed by DUBs from these proteins or cleaved into single moieties before it can modify substrates (Verma et al. 2002; O'Donoghue & Gordon 2008). Substrates also need to be deubiquitinated to turn off a signalling pathway or to allow them to to enter the proteasome, which is carried out by the proteasome subunit Pad1(O'Donoghue & Gordon 2008).

1.4.4 Ubiquitin Like Proteins

After the discovery of ubiquitin other similar proteins were found and classed as UBiquitin Like proteins (UBL). Many were discovered through sequence homology and all share ubiquitin's β-grasp fold tertiary structure. These modifiers are attached to target proteins via a similar cascade and signal for different pathways or regulate proteins. Although several UBLs have been identified, two of the most well studied are Neural

precursor cell Expressed and Developmentally Down-regulated 8 (Nedd8) and Small Ubiquitin-like MOdifier (SUMO).

Nedd8 share ~55% homology to ubiquitin and has its own dedicated E1 and E2's but as yet no specific E3 has be identified for Nedd8 (Kerscher et al. 2006). Nedd8 is attached to conserved lysine residue of E3 cullins subunits. This neddylation appears to control the interaction between E3s and E2s and, therefore, neddylation of cullins activates E3 ligases (Hori et al. 1999; Chew & Hagen 2007). It is believed that Nedd8 has other substrates, obesides cullins, and therefore performs other functions which have yet to be identified (Kerscher et al. 2006).

SUMO shares ~18% homology to ubiquitin and has its own E1, E2s and E3s (Kerscher et al. 2006). As with Nedd8, a similar situation exists for SUMO, since some E2s are SUMOlated and SUMOlation is also needed for APC/C to degrade securin and cyclinB, but its precise function remains elusive (Dieckhoff et al. 2004).

1.4.5 Ubiquitin Binding Domains

Ubiquitin, and its different chain types, need to be recognised and distinguished in order to perform their function. This is carried out by several Ubiquitin Binding Domains (UBD) including Ubiquitin Interacting Motif (UIM) (Deveraux et al. 1994; Beal et al. 1996), UBiquitin Associated (UBA) domain (Wilkinson et al. 2001), Pleckstrin-like Receptor for Ubiquitin (Pru) domain (Husnjak et al. 2008), Coupling of Ubiquitin conjugation of Endoplasmic reticulum degradation (CUE) domain, Npl4 Zinc Finger (NZF) domain, GRAM-Like Ubiquitin binding in Eap45 (GLUE) domain, GGA and Tom1 (GAT) domain, Polyubiquitin-Associated Zinc binding (PAZ) domain, Vps-27, HRS and STAM (VHS) domain and the Ubiquitin E2 Variant (UEV) domain (Hicke et al. 2005; Haglund & Dikic 2005). These UBDs are found in proteins responsible for transporting ubiquitinated substrates or in proteins that need to bind to ubiquitin to carry out their function such as DUBs and proteasome subunits (Hicke et al. 2005).

These domains take on a variety of different structures (Figure 3). The UIM domains consists of a single α helix whereas the CUE domain and the UBA domain consists of

three consecutive α helices. The Pru domain takes on a Pleckstrin-Homology Domain (PHD) β-sandwich fold of four anti parallel β-sheets that pack against another strand of three β-sheets. The GLUE domain takes on a part PHD fold with a C-terminal helix (Schreiner et al. 2008).

Studies investigating how these UBDs recognise ubiquitin revealed that, despite their different structures, nearly all of these domains bind to ubiquitin through the same area around its isoleucine 44 hydrophobic patch (Figure 3) (Haglund & Dikic 2005).

Binding studies of isolated domains show that the same subtypes of UBD display preferences for different chain types. This has lead to the definition of four classes for UBA domains. Class 1: are UBA domains that selectively bind K48 linked chains. Class 2: contains UBAs that show a preference for K63 linked ubiquitin chains. Class 3: do not bind to ubiquitin chains. Class 4: bind ubiquitin chains regardless of the linkage (Raasi et al. 2005).

Ohno et al. (2005) demonstrated that the UBA domain of Dsk2 interact with ubiquitins Ile-44 patch through a hydrophobic pocket. This pocket was formed by the loop1 between the α1 and α2 helices and the C-terminal of the α3 helix. In particular, Met-342 of loop1 formed a hydrogen bond with Gly-47 of ubiquitin causing it to fit tightly inbetween ubiquitins β3 and β4 strands. Further work by Jean-François Trempe et al. (2005) on *S. pombe* Mud1 UBA domain went on to show that along with this primary binding site, there is a secondary ubiquitin binding site formed by residues 309-320 and 331 between α2 and α3. They hypothesised that when two or more ubiquitin molecules formed chains via K48 linkage, this gave rise to a confirmation where one ubiquitin could bind the primary site, and the other could bind the secondary site. This would make the UBA domain select for K48 chains, and would explain why mutating Phe-330 of the secondary site caused a reduction in Ub_2 K48 but not monoubiquitin binding.

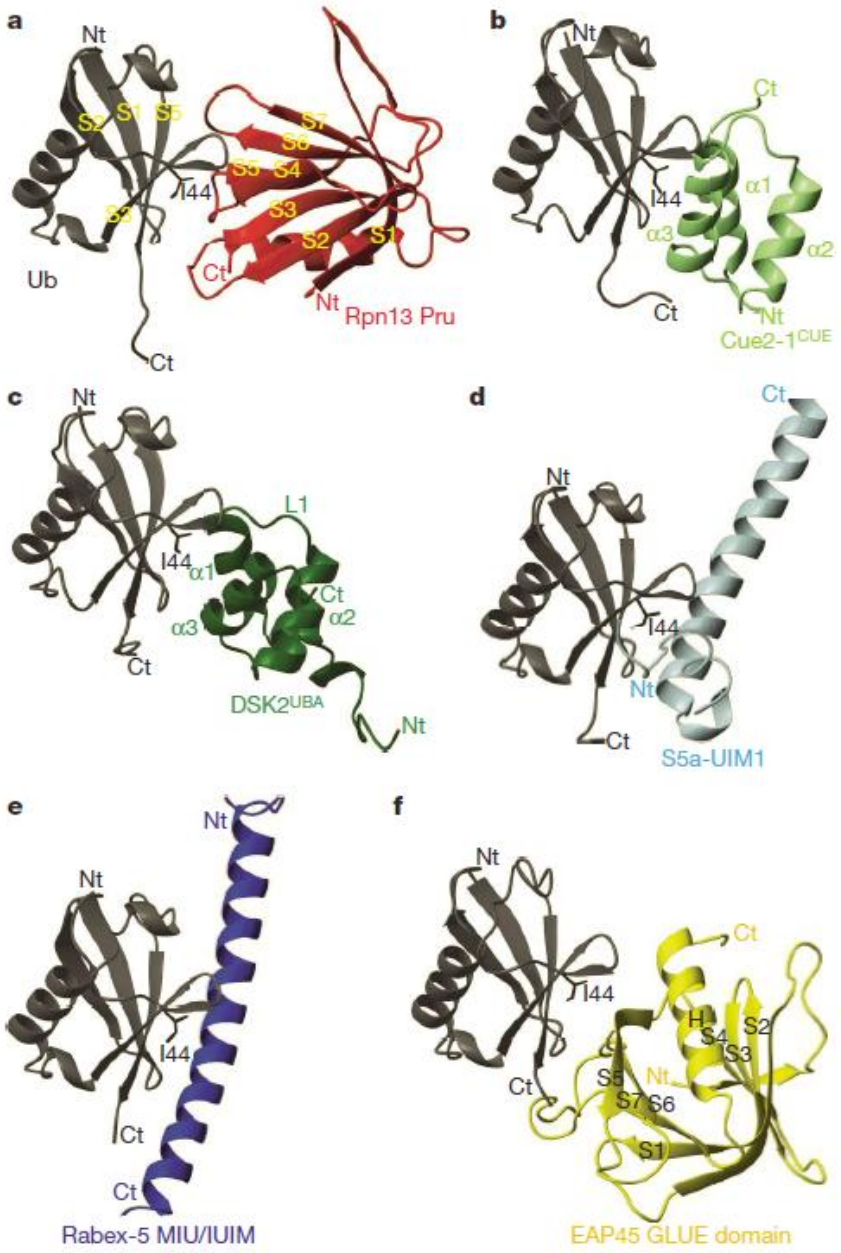


Figure 3: Tertiary Structures of UBDs.

Taken from Schreiner et al. (2008), cartoon representation of UBDs: **a)** Pru domain of Rpn13 **b)** α helices of CUE domain from Cue2 protein **c)** three α helices of DSK2s UBA domain **d)** single α helix of S5as UIM1 domain **e)** single α helix of Rabex-5 IUIM and **f)** GLUE domain of EAP45 interacting with ubiquitin (grey) at isoleucine 44 patch.

1.5 E3s & APC/C

1.5.1 Ubiquitin Ligases

E3 ubiquitin ligases are responsible for recognising and transferring ubiquitin from an E2 to a specific target protein. To do this there are hundreds of different E3s which can be classed into 3 groups depending on whether they contain a Homologous to E6-AP Carboxyl Terminus (HECT) domain, the Really Interesting New Gene (RING) finger and UFD2-Homology Domain (U-box) (Pickart 2001; Nakayama & Nakayama 2006).

The different sub-classes act in different ways. E2s transfer ubiquitin to HECT E3 ligases which contain conserved cysteines residues capable of forming covalent bonds to ubiquitin before transferring it to its substrate. However RING and U-box E3s act as a scaffold to hold the E2 and the substrate in the correct orientation, allowing the ubiquitin to be pass directly from the E2 without binding the E3 first (Figure 2).

RING type E3s are the largest family of E3s and contain two of the most important RING E3s. The SKP1–CUL1–F-box-protein (SCF) and Anaphase Promoting Complex/Cyclosome (APC/C) are multimeric ligases involved in the UPS which are made of four components: i) a RING-finger containing protein which contain conserved cysteine and histidine residues. The RING protein interacts with the E2 and facilitates the movement of ubiquitin, ii) a scaffold protein such as a cullin, iii) a protein which recognises specific substrates; for example an F-box protein. F-box proteins can also be sub-classed into three types: WD40 repeats (FBXW); leucine-rich repeat (FBXL) and other (FBXO) and iv) an adaptor protein which recognises F-box proteins (Bai et al. 1996; Pickart 2001; Nakayama & Nakayama 2006).

1.5.2 APC/C

One of the most well studied RING type E3 ligases is the Anaphase Promoting Complex/Cyclosome (APC/C), an important target of the spindle assembly checkpoint (Bastians et al. 1999; Jallepalli et al. 2001). In fission yeast APC/C is a 1.5MDa multimeric complex made of 13 core subunits (Table 1) and two co-activators Slp1 and

Ste9/Srw1 (commonly known as Cell Division Cycle 20 (Cdc20) and Cdh1/Hct1 respectively) which are responsible for recognising substrates (Schwab et al. 1997; Fang et al. 1998). The catalytic core of fission yeast APC/C is made up of Apc11 and Apc2. Apc11 is a RING finger containing protein which, in humans, interacts with two E2s, UbcH10 (fission yeast Ubc11) and UbcH5 (Ubc4) (Rape & Kirschner 2004; Pickart & Fushman 2004). Apc2 is a cul1-related protein which acts as a scaffold and interacts with Apc11 (Gmachl et al. 2000; Tang et al. 2001). The subunits Nuc2, Cell Untimely Torn 9 (Cut9) and Cut23 act as adaptor proteins. They contain TrichotetraPeptides Repeats (TPR) motifs which bind to C-box domains and isoleucine-arginine dipeptide (IR) tails of the two co-activators. The co-activators can recognise substrates containing Destruction boxes (D-box) RxxLxxxxN and KEN-boxes KENxxxxN through WD40 domains (Lamb et al. 1994; Burton & Solomon 2001; Yamano et al. 2004; Burton et al. 2005; Kraft et al. 2005). Many of APC/Cs subunits do not have a catalytic role, but it is believed that they may be important in giving APC/C its "platform and arc lamp" 3D structure (Figure 4) (Passmore & Barford 2005).

Although APC/C is active throughout the cell cycle and has many substrates, its most important role, from which it gets its name, is controlling the transition to anaphase from metaphase (Figure 5) (Bastians et al. 1999; Jallepalli et al. 2001). In *S. pombe* APC/C^{Slp1} ubquitinates Cut2 (commonly called securin) which acts as an inhibitor of the enzyme Cut1 (seperase).When Cut2 is degraded, Cut1 is then free to cleave the Rad21 (SCC1) subunit of the cohesion complex that holds the sister chromatids together as chromosomes. After this cleavage, the two sister chromatids can separate and migrate to different ends of the cell to form two nuclei and complete mitosis (Chang et al. 2001).

1.5.3 APC/C Co-Activators

APC/Cs two co-activators bind to the ligase at different points of the cell cycle and are responsible for recognising different substrates at specific times. APC/C^{Cdc20} forms at G_2

S. pombe	*S. cerevisiae*	*C. elegans*	*D. melanogaster*	*Vertebrates*	*Function*
*Nuc2	Cdc27	MAT-1/POD-5	CDC27	APC3/Cdc27	tetratricopeptide (TPR) motif, binds Cdc20/Cdh1
*Cut4	APC1	MAT-2/POD-3	Shattered	APC1/Tsg24	Binds APC11
*Cut9	Cdc16	EMB-27/POD-6	CDC16	APC6/Cdc16	TPR motif, binds Cdh1
*Cut20	Apc4	EMB-30	CG4350	APC4	
*Cut23	Cdc23	MAT-3/POD-4	CG2508	APC8/Cdc23	TPR motif
Apc2	Apc2	K06H7.6	Morula	APC2	Cullin Related
Apc5	Apc5	M163.4	IDA	APC5	
Apc10	Doc1	F15.H10.3	CG114190.3	APC10/DOC1	Substrate recognistion
Apc11	Apc11	F35G12.9	Lemming	APC11	RING, E2 recruitment, E3 activity
Apc13	Swm1	-	APC13	APC13/SWM1	
Apc14	-	-	-	-	
Apc15	Mnd2	-	-	-	
*Hcn1	Cdc26	-	-	Cdc26	
-	-	-	CG14444 (?)	APC7	TPR motif, binds Cdc20/Cdh1
*Slp1	Cdc20	Fzy-1	Fzy	Cdc20	WD40, substrate recognition
Ste9/Srw1	Cdh1	Fzr-1	Fzr	Cdh1/Hct1	WD40, substrate recognition
-	Ama1	-	-	-	WD40, substrate recognition
-	-	-	Rap	-	WD40, substrate recognition
-	-	-	Cortex	-	WD40, substrate recognition

Table 1: Subunits of APC/C.

Subunits that comprise the APC/C complex in *S.pombe* and its homologues in *S. cerevisiae, C. clegans, D. Melanogaste*r and vertebrates. * denotes essential genes in fission yeast. Co-activators are shown in the bottom box.

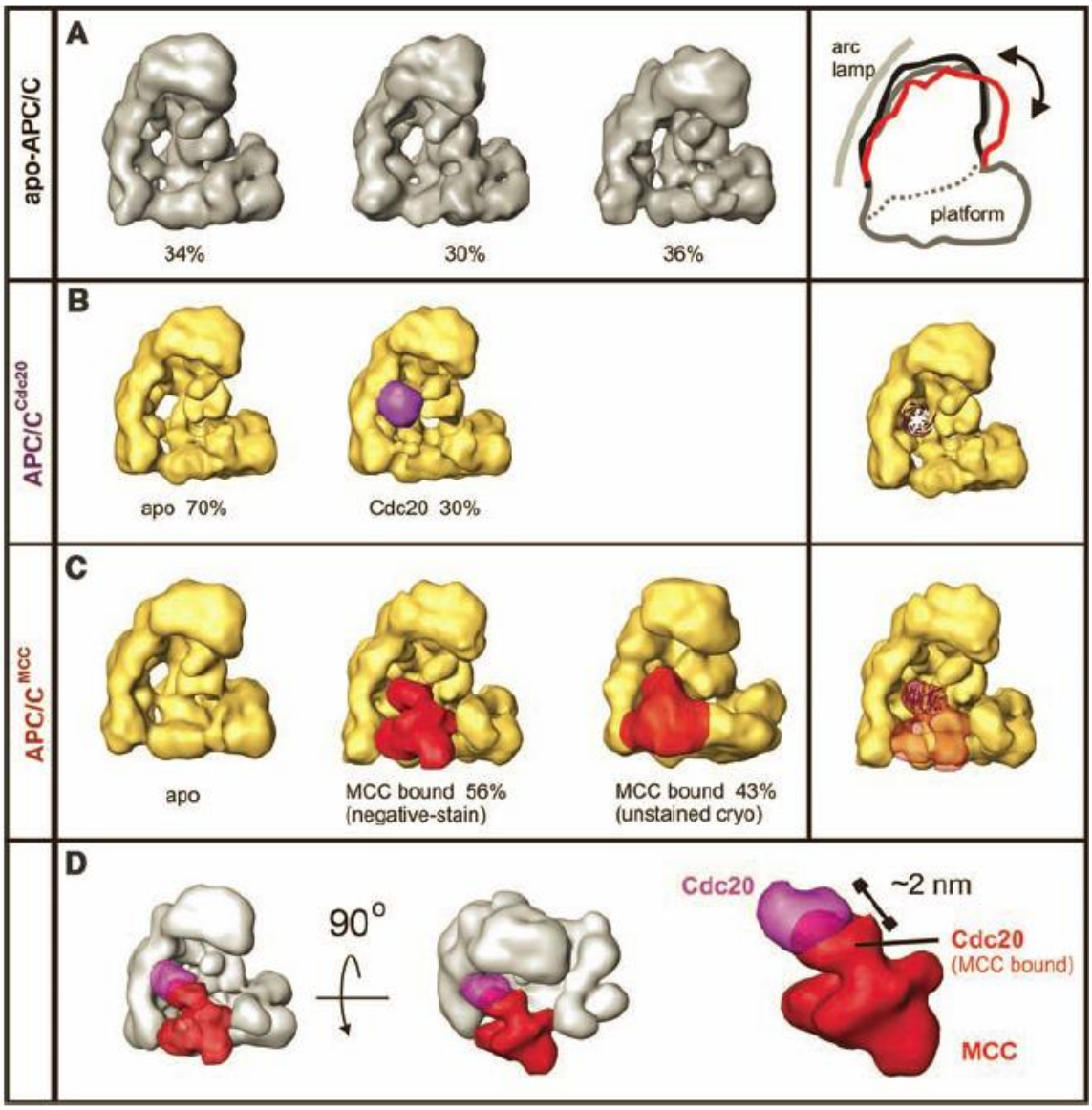


Figure 4: Structure of APC/C

Strucuture of APC/C obtained from EM. Panel **B** shows APC/C^{CDC20}, panel **C** shows APC/C^{MCC} whilst panel **D** show the superposition of CDC20 and MCC on APC/C (Passmore et al. 2005)**.**

but its activity is suppressed by the Mitotic Checkpoint Complex (MCC). The MCC is a complex which binds to Cdc20 via a subunit called the Mitotic Arrest Deficient 2 (Mad2). The MCC inhibits APC/C until all chromosomes are bound to spindles, at which point it dissociates and allows APC/C^{Cdc20} to act on substrates containing D-boxes, many of which are associated with the onset of anaphase, such as securin (Cut2 in *S. pombe*) (Acquaviva & Pines 2006; Nakayama & Nakayama 2006). Around anaphase, unbound Cdh1 is dephosphorylated by Cdc14, which allows Cdh1 to bind to APC/C to form APC/C^{Cdh1}. APC/C^{Cdh1} recognises both D-boxes and KEN boxes causing ubiquitination of substrates involved in the cell cycle, such as CyclinA and CyclinB. APC/C^{Cdh1} also recognises Cdc20 and causes it to be degraded after anaphase. At late G_1 to S phase Cdh1 is phosphorylated by Cdk1-CyclinB causing Cdh1 to dissociate from APC/C and remain inactive until anaphase (Pfleger & Kirschner 2000; Peters 2006; Reddy et al. 2007; Skaar & Pagano 2008).

1.5.4 Regulating APC/C Activity

Unlike some RING type E3s, APC/C can always recognise its substrates, but its activity is regulated by phosphorylation and the MCC (Shteinberg et al. 1999; Kramer et al. 2000; Rudner & Murray 2000; Kraft et al. 2003). Phosphorylation can both activate and inhibit APC/C activity, depending on the kinases and the subunit it phosphorylates. For example, in fission yeast phosphorylation of Cut4 and Nuc2 by PKA inactivates APC/C (Yamada et al. 1997; Kotani et al. 1998; Acquaviva & Pines 2006). However, phosphorylation by Cdk1-CyclinB (homologous to *S pombe* Cdc2-Cdc13) and Polo Like Kinase 1 (Plk1) (Plo1 in *S. pombe*) causes activation. In other organisms, these kinases have been shown to act on Cdc27 (*S. pombe* Nuc2), Cdc23 (Cut23) and Cdc16 (Cut9) and that these TPR containing subunits need to be phosphorylated in order to bind co-activators (Rudner & Murray 2000; Golan et al. 2002; Kraft et al. 2003). Studies in fission yeast have shown that phosphorylation of Hcn1 by Cdc2-Cdc13 is required by cells to carry out the final stages of mitosis (Yoon et al. 2006). The phosphorylation of Cut9 has also been indicated to be important for regulating APC/C activity. Whole cell extracts from a strain in which endogenous *cut9+* has been replaced with a HA tagged

gene revealed that the Cut9 protein exists in two different molecular weight forms when probed by Western blot. Further analysis concluded that the heavier molecular weight version of Cut9 was due to phosphorylation of the protein, and this phosphorylated form was only present in the APC/C complex at metaphase (Yamada et al. 1997).

The type of ubiquitination performed by APC/C may also be regulated by the E2 it interacts with. For example, in budding yeast it has been observed that the E2 Ubc4 causes mono-ubiquitiation of substrates, whilst the E2 Ubc1 causes poly-ubiquitination (Rodrigo-Brenni & Morgan 2007).

The MCC is a multimeric complex (described in detail in section 1.6) which regulates APC/Cs activity (Sczaniecka et al. 2008). During metaphase, chromosomes are connected through their kinetochores to two spindle microtubules radiating from each end of the cell. This process is essential for the equal segregation of DNA into each new cell. The MCC is formed and loaded onto APC/C only in the presence of unbound kinetochores. The complex inhibits APC/C activity by binding to Slp1/Cdc20 which prevents it from interacting with substrate. This ensures that the cell does not enter anaphase until all the chromosomes have been completely attached to spindles (Yang et al. 2008).

1.5.5 Mis-Regulation of APC/C and Disease

Although inactivation of APC/C is lethal, temperature sensitive (*ts*) mutants of essential APC/C subunits have been constructed in fission yeast. Shifting these mutants to a restrictive temperature results in mis-regulation of APC/Cs activity and generates a distinctive phenotype caused by the failure to degrade Cut2 (securin) (Samejima & Yanagida 1994). As a consequence, Cut1 (separase) cannot cleave the cohesion complex holding the two sister chromatids together but the cells continue into anaphase. This gives rise to the Cell Untimely Torn (Cut) phenotype which appears in two forms. In the less severe case, the septa forms and severs the nucleus, splitting the DNA unequally between the daughter cells. In the more severe phenotype, all the DNA moves to one daughter cell giving rise to a cell with no DNA, and another which is polyploid (Toda et

al. 1981; Hirano et al. 1986; Yamashita et al. 1999). Since APC/C is also responsible for regulating cyclin levels, which control passage from metaphase to anaphase, it was not understood how disruption to APC/Cs activity allowed cells to carry on through to anaphase. Chang et al. (2001) showed that these mutations are hypomorphic since cyclin degradation is not affected in *cut* mutants.

Since APC/C is a major component in regulating the cell cycle it is not surprising that it has been linked to many diseases. Mis-regulation of APC/C has a role in several types of cancer (Wang et al. 2003; Nakayama & Nakayama 2006). As stated above, deactivation of APC/C can cause aneuploidy, whereas APC^{Cdh1} has been shown to be particularly important for maintaining human genomic stability (Engelbert et al. 2008). However, up regulation in APC/C activity can also lead to cancer, as seperase needs a small amount of securin in order to function (Jallepalli et al. 2001). This makes APC/C and the UPS attractive targets for drug development. A proteasome inhibitor, called Bortezomib (marketed as Velcade), was approved by the FDA in 2003 and recommended for use in the UK by NICE in 2006, to treat relapsed and refractory multiple myeloma. In light of this drug approval, other proteasome inhibitors have been suggested as cancer treatments, including Salinosporamide A which is currently in trails (Feling et al. 2003; Nakayama & Nakayama 2006). However, it is now believed that proteasome inhibitors are specific to cancers of B cells as they increase stress put on the ERAD pathway and as a result cause the cells to die. Therefore, this type of treatment may not work on other forms of cancer (personal communication Rasmus Hartmann-Petersen).

1.6 Spindle Assembly Checkpoint

Separate from the DNA damage checkpoint, is another important mitotic checkpoint called the Spindle Assembly Checkpoint (SAC). This checkpoint occurs at the junction between metaphase and anaphase, and prevents cells from committing to nuclear division before they are ready. This is achieved by regulating APC/C^{Slp1} activity via the MCC (Figure 5). The MCC is normally made of Budding Uninhibited by Benzimidazole

3 (Bub3), Mitotic Arrest Deficiency 2 (Mad2) and Mad3. However, in *S. pombe*, Bub3 is not present in the MCC as Mad3 lacks a sequence needed to bind Bub3 which is present in other organisms (Sczaniecka et al. 2008). The MCC can detect when kinetochores are not bound to a microtubule, causing the MCC to be loaded onto Slp1 attached to APC/C and blocks its ability to recognise substrates (Chung & Chen 2003; Burton & Solomon 2007). The MCC is constantly being removed from APC/C ^{Slp1} but, as long as a kinetochore remains unbound, the complex will be loaded onto APC/ C ^{Slp1} to maintain the inhibition. When all the kinetochores are bound, MCC dissociates from APC/C ^{Slp1} causing it to re-activate and degrade Cut2 leading to the onset of anaphase (Bharadwaj & Yu 2004).

1.6.1 Mad2

Mad2 exists in two different states, open (O-Mad2) and closed (C-Mad2). When a kinetochore is unbound it can be recognised by Mad1 which binds to it. O-Mad2 cannot directly bind to Mad1, as it is in a kinetically higher energy confirmation, but C-Mad2 can. O-Mad2 binds to C-Mad2 in a C-Mad2-Mad1 dimer and is converted into an intermediate I-Mad2. The I-Mad2 then converts into C-Mad2 and dissociates from the C-Mad2-Mad1 dimer where it is then able to bind Mad1 or APC/C^{Slp1} via Slp1. Mad2 bound to Slp1 then recruits other components of the MCC to inhibit APC/C^{Slp1} (Yang et al. 2008).

Mad2 is phosphorylated at its C-terminus at several different serine residues. These modifications cause Mad2 to revert to its open confirmation preventing it from binding to Cdc20 and therefore cause Mad2 and the MCC to dissociate from APC/C (Wassmann et al. 2003; Kim et al. 2010). In addition the protein Cmt2/p31^{COMET} can bind to C-Mad2 bound to APC/C^{Cdc20} and causes Cdc20 to be auto-ubiquitinated and degraded. This then removes the inhibition of the MCC and when a new Cdc20 protein attaches to APC/C it is free to act on its substrates. Cmt2/p31^{COMET} also prevents to O-Mad2 converting to C-Mad2, by binding C-Mad2 in a dimer with Mad1, blocking it from binding O-Mad2. This consequently prevents C-Mad2 from being loaded onto APC/C (Luo & Yu 2008).

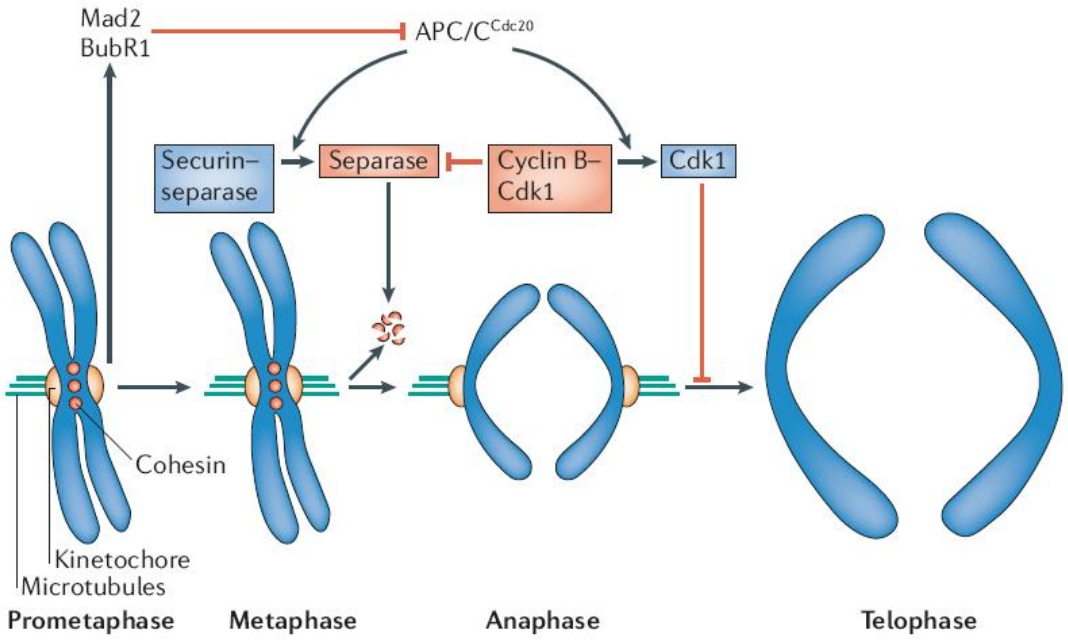


Figure 5: Schematic Diagram of the Spindle Checkpoint.

During metaphase chromatids are bound to each other by a cohesin complex to form chromosomes. The chromosomes are attached to microtubules via kinetochore on each chromatid. Until all kinetochores are bound, APC/C^{Cdc20} is inhibited by the MCC complex. When all of the kinetochores are bound, APC/C^{Cdc20} is released from its inhibition and can act on its substrate securin. When securin is ubiquitinated and degraded, the enzyme separase is released to cleave the cohesin complex holding the sister chromatids together. The cell can then progress to anaphase where the sister chromatids move to separate poles of the dividing cell (Peters 2006).

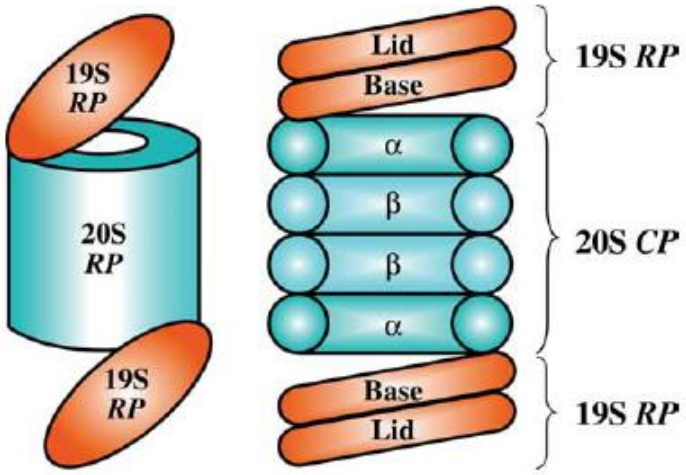
1.7 26S Proteasome

The 26S proteasome is a 2.5mDa multi-subunit complex made up of a 19S regulatory particle and a 20S catalytic particle which degrade proteins in an ATP-dependent manner (Figure 6). In most organisms the proteasome is found in both the nucleus and cytoplasm (Hoffman et al. 1992; Hartmann-Petersen et al. 2003), except in fission yeast where it is concentrated at the nuclear periphery (Wilkinson et al. 1999).

1.7.1 19S Regulatory Particle

The 890 kDa 19S regulatory particle is found at either end of the proteasome. Under high salt conditions it can be sub divided into a lid and a base, which makes contact with the 20S core in the 26S complex (Miller & Gordon 2005; Bohn et al. 2010). The lid is made of non ATPase subunits: Rpn3 (*S. cerevisiae* Rpn3), Rpn5a/b (Rpn5), Rpn6 (Rpn6), Rpn7 (Rpn7), Rpn8 (Rpn8), Rpn9 (Rpn9), Pad1 (Rpn11), Mts3 (Rpn12), and Rpn15/Dss1 (Rpn15/Sem1) (Wang et al. 2007; Jung et al. 2009; Bohn et al. 2010). The Pad1 subunit is a deubiquitinase which removes ubiquitin from substrates to be recycled and allows the substrate to fit into the proteasome (Verma et al. 2002).

The base contains a class of ATPases known as the ATPases Associated with various cellular Activities (AAA) ATPases. These reverse chaperones utilise the energy they obtain from converting ATP to ADP, to unfold the deubiquitinated substrates before they enter the catalytic core. In fission yeast there are six AAA ATPases: Rpt1 (*S. cerevisiae* Rpt1), Mts2 (Rpt2), Rpt3 (Rpt3), Rpt4 (Rpt4), Pam2 (Rpt5) and Rpt6 (Rpt6) (Glickman et al. 1998; Navon & Goldberg 2001; Wang et al. 2007; Jung et al. 2009; Bohn et al. 2010). In addition Mts2 and Pam2 control entry into the 20S to prevent non specific degradation of proteins. They do this by controlling the N-terminus of α subunits which can move and block the central pore of the 20S (Braun et al. 1999; Unno et al. 2002; Bohn et al. 2010).


19S
RP
20S
RP
19S
RP
Lid
Base
α
β
β
α
Base
Lid
19S RP
20S CP
19S RP

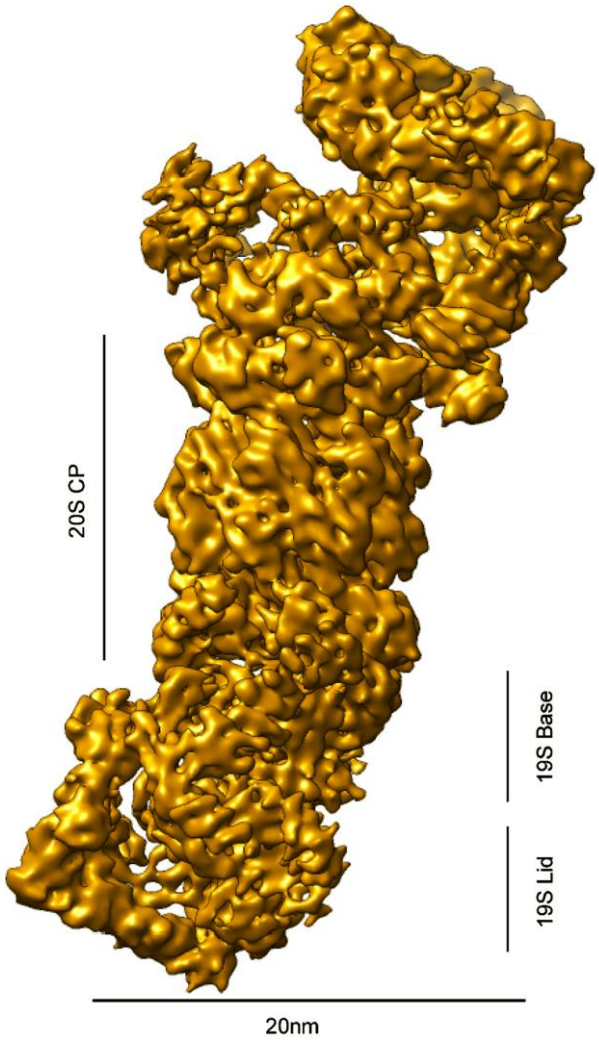

20S CP
19S Base
19S Lid
20nm

Figure 6: Organisation of the 26S Proteasome.

The top panel shows a cartoon depiction of the 26S proteasome which can be divided into a 19S Regulatory Particle (RP) and a 20S Core Particle (CP). The RP comprises of a lid and base which contains subunits which recognise ubiquitin chains and interact with shuttle factors. It is also responsible for deubiquitinating and unfolding proteins before they enter the core. The RP also regulates entry of proteins into the CP to prevent unspecific proteins being degraded. The 20S CP is made up of four rings, each containing seven subunits. The two internal rings are comprised of β subunits whilst the rings that cap either end of the core are made of α subunits. These rings form a cylinder with an inner pore in the middle through which proteins pass and are degraded. (Miller & Gordon 2005). The bottom panel shows the high resolution (9.1 and 6.7 Å at FSC of 0.5 and 0.3) structure of *S. pombe* 26S proteasome (Bohn et al. 2010).

The base also performs other functions, using its non ATPases subunits: Mts4 (Rpn1), Rpn2 (Rpn2), Pus1 (Rpn10) and Rpn13a/b (Rpn13). Rpn2, Mts4 and Pus1 interact with Mts5 and therefore join the lid and base together (Wilkinson et al. 1999). Rpn2 is a regulator that not only binds Rpn13 but together with Mts4 acts as scaffold proteins (Finley 2009). Mts4 can recognise the Ubl domains of shuttle proteins (see section 1.81.8) (Elsasser et al. 2002; Seeger et al. 2003). Pus1 and Rpn13 act as ubiquitin receptors; although Pus1 can also act independently from the proteasome as a shuttle protein and can bind both the base and lid via Mts4 and Mts3 (Glickman et al. 1998; Wilkinson et al. 2000; Seeger et al. 2003; Husnjak et al. 2008; Schreiner et al. 2008; Bohn et al. 2010). The interaction between Pus1 and Mts3 requires the Pus1 vWA domain but is enhanced by Mts3 binding to the UIM of Pus1 (Riedinger et al. 2010).

1.7.2 20S Core Particle

The 720 kDa 20S catalytic particle is made up of 14 subunits that form a stack of two α and two β heptameric rings. The β rings reside in the middle, with an α ring at either end. This conformation forms a cylinder 15nm long and 11 nm wide with a 5nm central pore through the middle. This pore can further be divided into three chambers, two in between the α and β rings and one main chamber formed by the β rings. The pore is just wide enough for a single unfolded substrate to pass through before being degraded into small peptides (Zwickl et al. 1999; Groll et al. 2000; Unno et al. 2002; Miller & Gordon 2005).

The two alpha rings reside at either end of the core and consist of seven α subunits: α1 SPBC646.16 (*S. cerevisiae* Prs2), α2 Pre8 (Pre8), α3 SPAC13C5.01c (Pre9), α4 Pre6 (Pre6), α5 Pup2 (Pup2), α6 SPAC6G10.04c (Pre5) and α7 Pre10 (Prs1). These subunits can bind proteins which regulate the proteasomes activity. The α rings are also responsible for controlling the gated entry into the 20S via the N-terminus of the α2, α3 and α4 subunits which can change their conformation to block the pore (Wang et al. 2007; Jung et al. 2009).

The two β rings occupy the middle of the core and are also made up of seven subunits: β1 Pre3 (*S. cerevisiae* Pre3), β2 Pup1 (Pup1), β3 Pup3 (Pup3), β4 SPAC31A2.04c (Pre1), β5 Pts1 (Pre2), β6 Pam1 (Pre7) and β7 SPBC577.10 (Pre4) (Wang et al. 2007; Jung et al. 2009). Each ring contains three subunits with proteolytic activity: i) β1 subunit which has peptidyl-glutamyl-peptidehydrolising-activity that recognises and cleaves at acidic residues, ii) β2 subunit which has trypsin-like activity which recognises and cleaves at basic residues and iii) β5 subunit which has chymotrypsin-like activity which recognises and cleaves at neutral residues. All three are members of the Ntn-hydrolase family and have an N-terminal active threonine residue which faces into the pore (Groll et al. 2000; Unno et al. 2002; Jung et al. 2009).

Purification and structural studies of *S. pombe* 26S proteasomes show that the level of α1-7, β1-7, Rpt1-6, Rpn1-3, Rpn5-9 and Rpn11-12 subunits remain constant, but the amounts of Pus1, Rpn13 and hydrolases Uch2 and Ubp6 vary due to their transient binding to the proteasome. The abundance of Rpn15/Dss1 is hard to determine since it only produced one unique peptide when analysed by mass spectrometry (Bohn et al. 2010).

1.8 Shuttle Factors

Ubiquitinated proteins that have been marked to be degraded need to be transported to the proteasome, which can be done in a variety of ways. Some E3s can interact with the proteasome and deliver ubiquitinated substrates directly to the proteasome (Xie & Varshavsky 2000; Fu et al. 2010). The proteasome itself contains subunits which possess UBDs which can interact with substrates directly (Miller & Gordon 2005; Schreiner et al. 2008). Another well studied mechanism are the shuttle factors which, in *S. pombe*, are known as Rhp23 (budding yeast Rad23), Pus1 (Rpn10) and Dph1 (Dsk2). These proteins have an N-terminal domain which can recognise and interact with the proteasome. Pus1 contains a von Willebrand factor A (vWA) domain where as Rhp23 and Dph1 contain an UBiquitin Like (Ubl) domain. They all also contain UBDs to

interact with ubiquitin. Pus1 has a UIM domain, Dph1 has a UBA domain and Rhp23 has two UBA domains (Wilkinson et al. 2001; Seeger et al. 2003).

These proteins share redundancy in *S. pombe* as single null mutants, of any of the three genes, are viable and do not display a phenotype. The two double mutants of *dph1*Δ*pus1*Δ and *dph1*Δ*rhp23*Δ are also viable, however, *dph1*Δ*rhp23*Δ strain display an elongated phenotype when grown 36^{o}C. In contrast, the *rhp23*Δ*pus1*Δ double mutant has a growth defect at 25^{o}C and is lethal at 36^{o}C due to excess strain put on the UPS. The triple mutant of *dph1*Δ*rhp23*Δ*pus1*Δ is never formed and is therefore synthetically lethal (Wilkinson et al. 2001).

1.8.1 Rhp23

The Ubl domain of Rhp23 has a similar structure to ubiquitin which allows it to be recognised by UBDs (Ryu et al. 2003; Mueller & Feigon 2003). The Ubl of Rhp23 and its budding yeast homologue Rad23, has been shown to bind the proteasome via the Mts4/Rpn1 subunit (Elsasser et al. 2002; (Seeger et al. 2003). This has lead to the hypothesis that after Rhp23 has taken an ubiquinated substrate to the proteasome, the Ubl domain binds to the UBA domains, causing Rhp23 to release its cargo (Rao & Sastry 2002).

Rhp23 is different from Pus1 and Dph1, as it contains not one but two UBA domains. One is internal and is denoted as UBA1, the other resides at the C-terminus of the protein and is denoted as the UBA2 domain. This structure is not only in yeast but also conserved in all eukaryotes (Mueller & Feigon 2002; Hartmann-Petersen et al. 2003). Although these two domains share a similar structure, they differ greatly in their sequences (Mueller & Feigon 2002). In human Rhp23 (hHR23A) UBA2 is a Class 2 UBA (refer to section 1.4.5) and preferentially binds to K48 chains where as UBA1 selects K63 making it a Class 1. However the Ubl-UBA1 fragment of the protein converts the UBA1 from a Class 2 to a Class 1 domain (Raasi et al. 2005). This would suggest that the domains in isolation might behave differently than when they are in the full length protein. Rhp23 has also been shown to interact with APC/C through its UBA

domains suggesting that Rhp23 binds ubiquitinated substrates attached to APC/C (Seeger et al. 2003).

1.8.2 Pus1

Pus1 is not only a shuttle factor but also a subunit of the proteasome (Wilkinson et al. 2000). It acts as an ubiquitin receptor allowing the proteasome to directly bind substrates. Another proteasome subunit that also acts as a receptor is Rpn13 which binds ubiquitin via its Pru domains. Unlike other organisms fission yeast contains two forms of Rpn13: Rpn13a and Rpn13b (Husnjak et al. 2008; Schreiner et al. 2008; Wilkinson et al. 2000).

Although Pus1 is a subunit of the proteasome, it can also exist as a free independent protein (Bohn et al. 2010). Pus1 binds to the proteasome via its N-terminal vWA domain. The vWA domain recognises the PC repeats of the proteasome Mts4, Rpn2 and APC/C subunit Cut4 (Seeger et al. 2003). Pus1 contains one UBD, a UIM at its C-terminus which can bind both K48 and K63 (Fu et al. 1998; Wilkinson et al. 2000; Miller & Gordon 2005; personal communication Colin Gordon). Studies show that mutating the conserved LAL(M)AL motif in the UIM to N can disrupt ubiquitin binding (Wilkinson et al. 2000).

1.9 Kinase Associated 1 Domains

Kinase Associated 1 (KA1) domains were first identified in the Microtubule Affinity-Regulating Kinase (MARK) family of kinases. They have since been identified in the C-terminus of other serine/threonine kinases. The domain is defined as being 50-100 amino acids in length and many domains end in a conserved glutamic acid, leucine, lysine, leucine (ELKL) motif which is believed to be structurally important. They also contain a conserved aspartic or glutamic acid residue (Figure 7) (Tochio et al. 2006; Townley & Shapiro 2007; Sanger 2010).

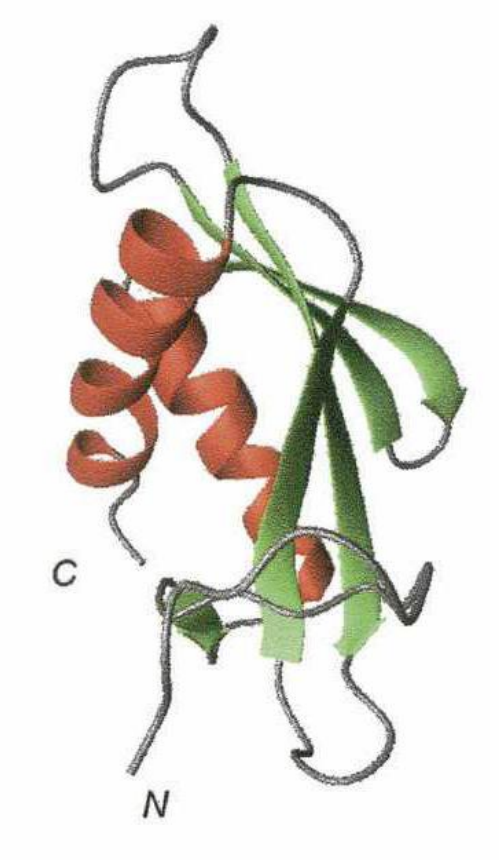


Figure 7: Tertiary Structures of KA1 Domain.

Cartoon representation of the structure of the KA1domain found in the MELK protein. Red residues fold to form α helices and green residues form β strands (Tochio et al. 2006)

The precise role of the KA1 domains has yet to be determined, but it has been suggested that they may have a role in localisation or autoregulation of the N-terminal kinase domain (Guo & Kemphues 1996; Beullens et al. 2005). Bioinformatics analysis, by Kay Hofmann (Miltenyi Biotec GmbH, Stoeckheimer), identified three *S. pombe* proteins which contain a KA1 domain: Ssp2 (SPCC74.03c), Ppk9 (SPAC23H4.02) and Kin1 (SPBC4F6.06). He also discovered a similar domain, which he named KA2 in the fission yeast Chk1 (SPCC1259.13) protein (Personal communication Kay Hofmann).

1.9.1 Kin1

Kin1 is the only *S. pombe* member of the MARK family of serine/threonine kinases (Levin & Bishop 1990; Tassan & Le Goff 2004). MARKs have various functions including controlling cell polarity, cell cycle, protein stabilisation and microtubule stabilisation. Most MARKs phosphorylate the KXGS motif in Microtubule Associated Proteins (MAP), such as Tau. MAPs bind to non mitotic microtubules via a domain which overlaps with the KXGS motif. The binding of MAPs causes the stabilisation of microtubules. However, when MAPs are phosphorylated this causes them to dissociate from the microtubules which then become unstable (Ebneth et al. 1999; Tassan & Le Goff 2004). MARKs have been implicated in neurodegenerative diseases since hyperphosphorylation of the MAP Tau causes it to form aggregates which are found in high concentrations in Alzheimer patients (Drewes et al. 1998; Tassan & Le Goff 2004; Trinczek et al. 1995). All MARK kinases have a C-terminal KA1 (with the exception of some splice variants) and many also have UBA domains (Tassan & Le Goff 2004; Tochio et al. 2006).

1.9.2 Ssp2 & Ppk9

Suppressor of Sts5 and Ppe1 mutants 2 (Ssp2) was identified in a screen trying to identify new genes involved in the regulation of growth polarity by looking for suppressors of *ppe1*Δ and *sts5 cs* mutants, which both have abnormal cell morphology (Matsusaka et al. 1995). Ssp2 is the *S. pombe* homologue of budding yeast Sucrose Non-Fermenting 1 (Snf1) and mammalian AMP-activated Protein Kinase (AMPK) α subunits (Celenza & Carlson 1984). Little was known about the Putative Protein Kinase 9 (Ppk9),

except that it is similar to Ssp2 in sequence, until Hanyu et al. (2009) showed that Ssp2 and Ppk9 are alternative α subunits of *S. pombe* AMPK.

Although Ssp2, Ppk9 and fission yeast AMPK still remain relatively unstudied, AMPK has been well studied in other organisms due to its involvement in diabetes and Wolff-Parkinson-White (WPW) syndrome which causes an irregular heartbeat (Hedbacker & Carlson 2008; OMIM 2011). AMPK is made up of three subunits: an α catalytic subunit, a β subunit which binds glycogens and holds the complex together and a γ subunit which regulates the α subunit (Dyck et al. 1996). In fission yeast AMPK consists of either Ssp2 or Ppk9 as α subunits, one known β subunit SPCC1919.03C/Amk2 and the γ subunit Cbs2 (Figure 8) (Townley & Shapiro 2007; Hanyu et al. 2009). This is different from the well studied *S. cerevisiae* AMPK, known as SNF1, which consists of one α subunit Snf1, one of three alternative β subunits Sip1, Sip2 or Gal83 and Snf4 the γ subunit (Sanz 2003). AMPK is a serine/threonine kinase that is involved in metabolic homeostasis. During time of low glucose, the γ subunit undergoes conformational changes which causes the α catalytic subunit to be activated. This allows AMPK to act on its substrates which then causes the transcription of glucose repressible genes as well as regulating activity of certain enzymes. These changes allow the cell to obtain energy from alternative carbon sources (Hardie et al. 1998; Hedbacker & Carlson 2008). Under times of cellular stresses such as heat shock, salt, pH and oxidative stress, AMPK has also been shown to be activated to maintain cell function (McCartney & Schmidt 2001; Sanz 2003; Hedbacker & Carlson 2008).

In budding yeast Snf1 has an Auto-Inhibitory Domain (AID), which is also found in mammalian homologues, that can bind to the N-terminal kinase domain and inhibit its activity (Crute et al. 1998; Hedbacker & Carlson 2008). The C-terminus of Snf1 can also bind Snf4 and the β subunits (Celenza et al. 1989). Studies also show that, removal of the C-terminus increases Snf1 activity and allows it to act independently from the β and γ subunits. Snf1 is phosphorylated at

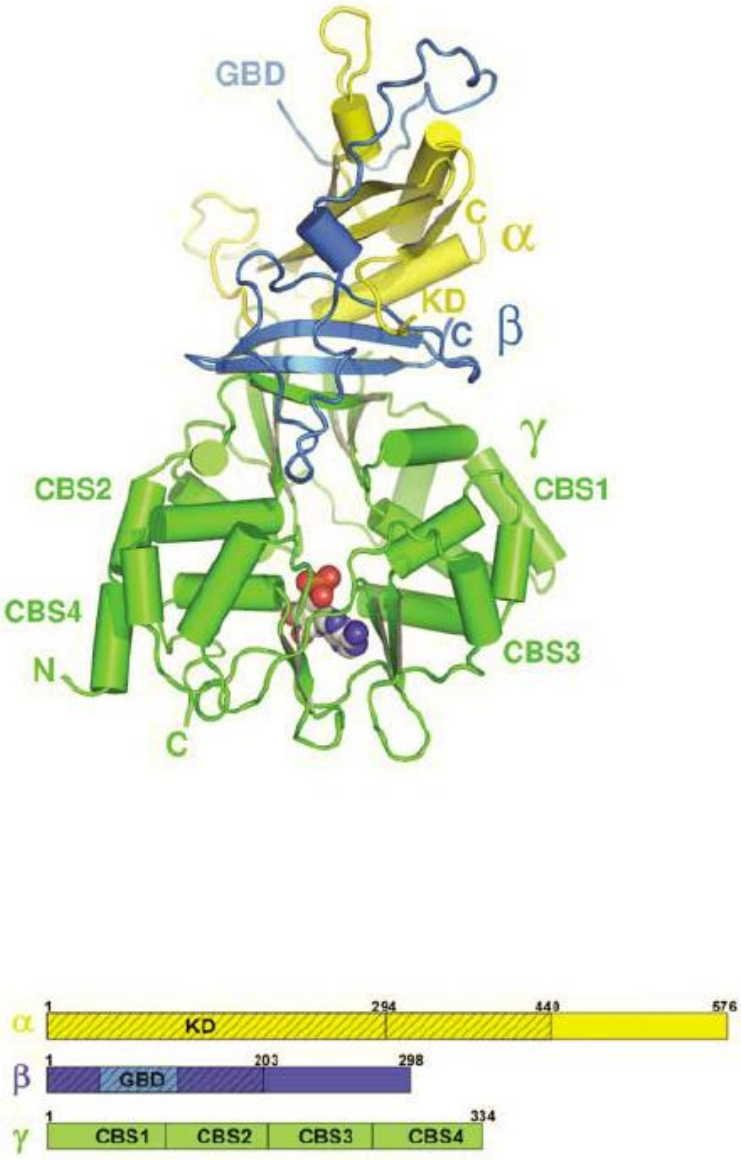


Figure 8: Structure of *S. pombe* AMPK.

The structure of AMPK from fission yeast. The α subunit is Ssp2 which has a N-terminal Kinase Domain (KD). The β subunit is SPCC1919.03C which has a Glycogen-Binding Domain (GBD) which allows it to interact with glycogens. Cbs2 is the γ subunit which contains four Cystathionine-Beta-Synthase domains (CBS) which bind adenosine derivates (Townley & Shapiro 2007).

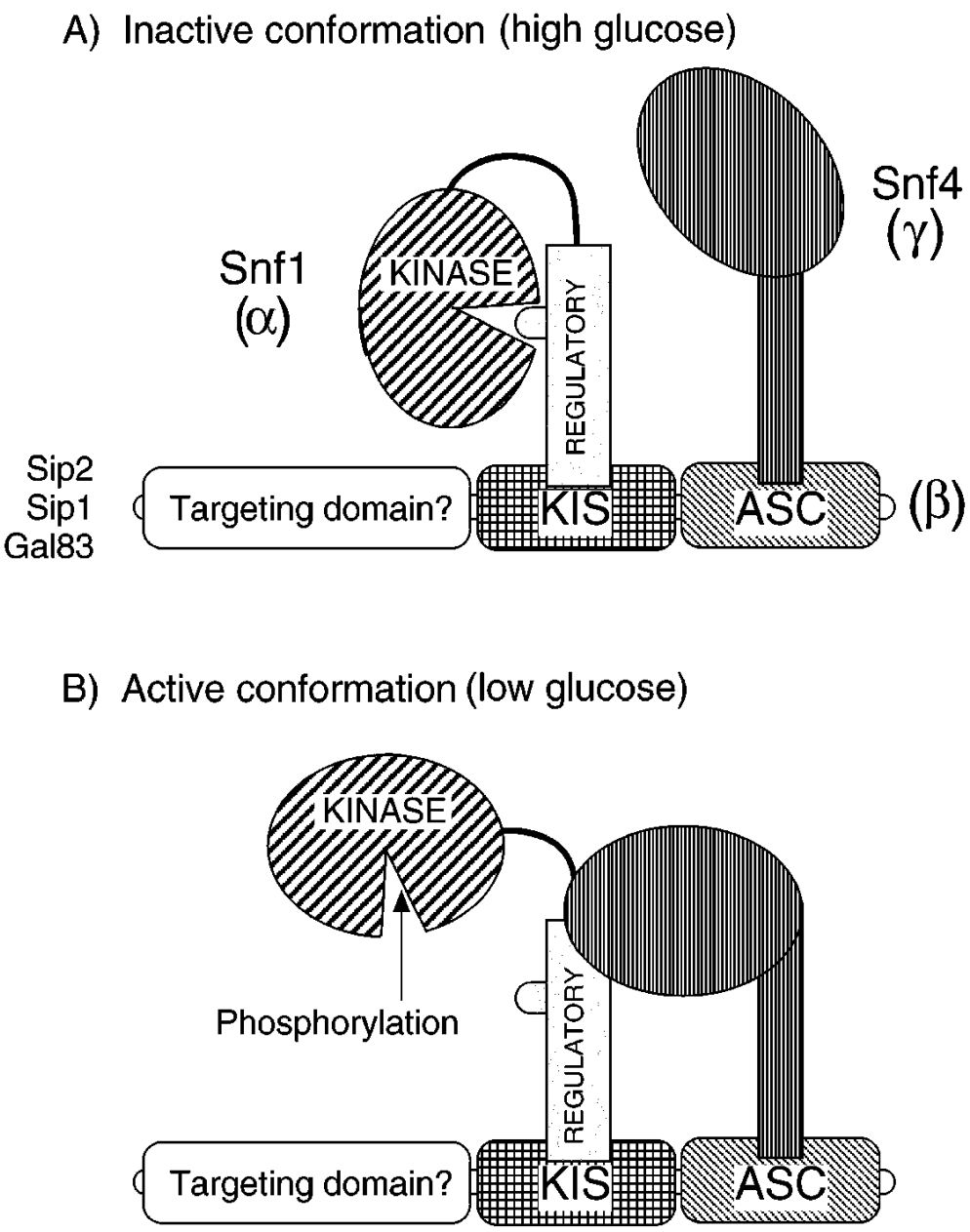


Figure 9: Regulation of SNF1 Activity in *S. cerevisiae*.

a) During times of high glucose the SNF1 complex is inactive. The Snf1 α catalytic subunit regulates its own kinase activity by binding to an Auto-Inhibition Domain (AID). The C-terminal of Snf1 binds to one of three β subunits via a Kinase Interacting Sequence (KIS) domain. The β subunits also bind the regulatory γ subunit Snf4 via another domain, the Associated with SNF1 kinase Complex (ASC). **b)** When glucose is limited, the SNF1 complex needs to be activated. This is achieved by Snf4 competing with Snf1 kinase domain for binding to the AID. This releases the kinase domain, which is also activated by phosphorylation and allows it to act on its substrates (Hardie et al. 1998).

Thr210 in its activation loop by upstream kinases which stimulates Snf1 kinase activation (Estruch et al. 1992; McCartney & Schmidt 2001). Snf4 is believed to regulate Snf1 activity by competing with the kinase domain of Snf1 for binding to the AID (residues 381-518) and therefore acts to antagonise auto-inhibition (Figure 9) (Amodeo et al. 2007; Hedbacker & Carlson 2008; Amodeo et al. 2010). Snf4 is also responsible for binding adenosine derivates, such as AMP and ATP, using four Cystathionine-Beta-Synthase (CBS) repeats of its two Bateman domains. Structural analysis suggests that these domains form a hydrophobic pocket that can bind AMP and ATP (Amodeo et al. 2007). It should be noted that, unlike AMPK, SNF1 is not activated by the presence of AMP. However, like AMPK it is inhibited by high concentrations of ATP which creates a negative feedback loop. There is also evidence that Snf4 can regulate the phosphorylation of Snf1, but how it does this has not yet been fully understood.

The β subunits share similarities in function, but signalling domains at their N-terminus cause them to occupy different cellular compartments during low levels of glucose. They bind the tip of the C-terminus of Snf1, in a region outside the AID via a C-terminal Kinase Interacting Sequence (KIS) domain and also bind Snf4 using an Associated with SNF1 kinase Complex (ASC) domain (Hardie et al. 1998; Hedbacker & Carlson 2008). They also contain Glycogen-Binding Domain (GBD) in the middle of the protein which interacts with glycogens (Amodeo et al. 2007).

The structure of the *S. pombe* AMPK complex containing Ssp2 has been solved, which revealed that Ssp2 has a C-terminal KA1 domain which appeared to interact with the β subunits (Jin et al. 2007; Townley & Shapiro 2007). It is also interesting to note that the sequence of Ssp2 suggests it contains a putative UBA domain (Hanyu et al. 2009).

1.9.3 Chk1

Chk1 is a highly conserved serine/threonine kinase which plays a role in DNA damage checkpoints. If DNA is damaged or fails to fully replicate, Chk1 is activated by phosphorylation by the *S. pombe* ATM/ATR Rad3 kinase (Capasso et al. 2002). This

sets off a cascade in which Chk1 phosphorylates Cdc25, causing it to be bound and sequestered by Rad24, the homologue of the 14-3-3 protein. Cdc25 is then not available to dephosphorylate Cdc2 attached to Cdc13, which is needed for activation of the Cdk-cyclin complex (Walworth et al. 1993; Furnari et al. 1997; Zeng et al. 1998). Chk1 also phosphorylates and stabilises the Cdc2 inhibitor Wee1 (O'Connell et al. 1997). This results in mitosis stalling until the problem can be rectified. During DNA damage, budding yeast Chk1 has been seen to phosphorylate the orthologue of securin, Pds1, which results in a stabilised protein which then cannot be ubiquitinated by APC/C (Xiao et al. 2005).

S. pombe Chk1 has a conserved kinase domain at its N-terminus which recognizes the motif Φ-X-β-X-X-(S/T) where Φ is a hydrophobic residue and β is a basic residue. The C-terminus contains a highly conserved domain within which a similar motif is found, but instead the phospho receptor has been replaced with a conserved aspartic acid residue (Palermo et al. 2008). This has lead to the hypothesis that this motif acts as a pseudo substrate and that the C-terminal of Chk1 may act as an auto-inhibitory domain for the N-terminal kinase. This is supported by the structure of the kinase domain and mutations studies which demonstrate that Chk1 kinase activity could be increased by point mutations in the C-terminal (Chen et al. 2000; Kosoy & O'Connell 2008). However, this is contradicted by other mutations which gave rise to loss of kinase function and yeast two hybrid analysis shows no interaction between the kinase domain and the C-terminus (Kosoy & O'Connell 2008). Never the less, it has been proven that the C-terminus of Chk1 is important for regulating kinase function, since removing the last 11 residues of the C-terminus is enough to render the kinase domain inactive (Kosoy & O'Connell 2008). It has also been shown that Chk1 regulates the MCC protein BubR1 (Mad3) (Zachos et al. 2007; Royou et al. 2005) and that it may also play a role in the spindle checkpoint in a Mad2-dependent manner (Collura et al. 2005).

1.10 Aims

Previous unpublished work from our group had identified interactions between APC/C and both Ssp2 and Ppk9. It has been hypothesised that this interaction may be due to the

KA1 domain of the two kinases. The aim of this project was to test this hypothesis and to further characterise the four KA-domain containing proteins and their influence on APC/C in *S. pombe* using both biochemical and genetic techniques.

The second part of this project was to enhance our understanding of the role the two UBA domains of Rhp23 play in the recognition of binding ubiquitin chains. The techniques employed looked closely at the UBA domains both as isolated moieties and within the full length of the Rhp23 protein. *in vitro* and *in vivo* assays were performed in order to study the UBA domains ubiquitin binding function and their role in the ubiquitin proteasome system.

Chapter 2
Material and Methods

2.1 General Methods

2.1.1 Sterilisation

Media and chemicals were sterilised by autoclave were appropriate by heating to 121^{o}C under pressure for 20mins. Otherwise chemicals were sterilised by passing through 0.22µm sterile membrane for small volumes or 0.22µm cellulose acetate 250ml flasks for larger volumes

2.1.2 Suppliers

All chemicals were supplied by Sigma whilst all biological reagents were supplied by Roche, GE Healthcare and Invitrogen unless stated otherwise.

2.2 Microbiological Methods

2.2.1 Solid & Liquid Media

All liquid media was made into solid media by addition of 2% (w/v) Bacto agar

2.2.2 Luria-Bertani (L-Broth):

1% (w/v) Tryptone

0.5% (w/v) Yeast extract

1% (w/v) Sodium chloride

2.2.3 Antibiotic Selection

Antibiotics were stored at -20^{o}C and added to sterilised media.

100µg/ml ampicilin and 50µg/ml kanamycin were used in both liquid and solid LB.

2.2.4 Transforming Bacteria

DNA was mixed with the volume of cells stated and incubated on ice for 5-30mins. Cells were then heat shocked in a water bath at 42^{o}C for time stated before being cooled on ice. L-broth was added and the cells allowed to grow at 37^{o}C for 1hr. The 1ml cultures were then spun and resuspended in 100µl which was spread on selective plate.

Cell Line	Volume	Time	Purpose
DB3.1	100µl	45 secs	Propagation of gateway entry vectors
Mach 1	50µl	30 secs	Propagation of plasmids
DH5α	50-100µl	45 secs	Propagation of plasmids
BL-21	50-100µl	2 mins	Protein induction

2.2.5 Growth Conditions

Bacteria were spread on solid media by use of sterile glass beads or sterile loops. All bacteria were grown on plates at 37^{o}C over night. Cultures were grown over night at 37^{o}C whilst shaking at 180rpm.

2.2.6 Storage of Bacteria

Bacteria were stored for short term on solid media at 4^{o}C. Stocks were stored long term at -80^{o}C in cryo tubes by adding 33.3% (v/v) glycerol to an overnight culture to make a final volume of 1ml.

2.3 *Schizosaccharomyces pombe*

2.3.1 *S. pombe* Strains

This study used strains derived from 972h^{+S} and 975h^{-L} using the standard background *leu1-32, ura4-D18, his3-D1, arg3 and either ade6-M210 or ade6-M216*

Ref No.	Genotype	Reference
101	*cdc25-22leu1.32h-*	Lab Stock
109	*cdc25-22leu1.32ura4D18h+*	Lab Stock
157	*mts2 ade6.216leu1.32ura4.D18 h-*	Lab Stock
648	*cut8 leu1-32 HA leu2 h-*	Lab Stock
858	*cut9::Cut9-3HALeu2+(leu1.32)h-*	Lab Stock
1011	*WT 5 Marker ade6-216h-*	Lab Stock
1012	*WT 5 Marker ade6-216h+*	Lab Stock
1013	*WT 3 Marker ade6-216h-*	Lab Stock
1014	*3 Marker ade6-216h+*	Lab Stock
1022	*3 Marker ade6-210h-*	Lab Stock
1023	*3 Marker ade6-210h+*	Lab Stock
1034	*rad3::uraleu-ade-h-*	Lab Stock
1295	*bub1::URA4leu1.32-ura4D-18h-*	Kevin Hardwick
1412	*Lid-TAP(kanR)ade6-M210leu1.32ura4-D18h-*	Kathy Gould
1531	*ssp2::G418 ade6 Ura4-D18 leu1.32 h+*	Bioneer Korea
1536	*ppk9::G418 5mk h+*	Bioneer Korea
1538	*ppk9::G418 5mk h-*	Bioneer Korea
1556	*h- ade6-210 leu1-32 ura4-DS/E [Ch16 ade6-216m23:: LEU2]*	Robin Allshire
1852	*chk1::HAade6-704leu1.32ura4-D18h+*	Lab Stock
1853	*chk1::Ura4+ade-leu1.32ura4-D18h+*	Lab Stock
1854	*chk1::Ura4+ade-leu1.32ura4-D18h+*	Lab Stocks
1952	*kin1::kanMY6 ade6.216 leu1.32 ura4-D18 h-*	Ken Sawin
1955	*kin1::kanMY6 ade6.216 leu1.32 ura4-D18 h+*	Ken Sawin
2039	*mad2::ura4 leu1-32 ade6M? ura4D-18 h-*	Dan Mulvihill
2466	*ssp2::NAT ade6 Ura4-D18 leu1.32 h+*	This Study
2468	*ppk9::HYG h-*	This Study
2478	*ppk9::HYG Kin1::Kan h+*	This Study
2479	*ppk9::HYG Kin1::Kan h-*	This Study
2480	*chk1::ura ppk9::HYG h+*	This Study
2481	*chk1::ura ppk9::HYG h-*	This Study
2482	*ssp2::NAT ppk9::HYG h+*	This Study
2483	*ssp2::NAT ppk9::HYG h-*	This Study
2484	*chk1::ura Kin1::Kan h+*	This Study
2485	*chk1::ura Kin1::Kan h-*	This Study
2488	*mts3.1 leu1-32 ura- ade- h+*	Lab Stock
2489	*mts3.1 leu1-32 ura- ade- h-*	Lab Stock
2503	*cut9-665 leu1.32 ura- h+*	Yanagida et al
2504	*cut9-665 leu1.32 ura- h-*	Yanagida et al
2505	*ssp2::NAT kin1::Kan h+*	This Study
2506	*ssp2::NAT kin1::Kan h-*	This Study
2507	*ssp2::NAT chk1::ura h+*	This Study

Ref No.	Genotype	Reference
2508	*ssp2::NAT chk1::ura h-*	This Study
2516	*ssp2::NAT kin1::Kan Chk1::ura*	This Study
2517	*ssp2::NAT ppk9::HYG kin1::Kan Chk1::ura h-*	This Study
2518	*ppk9::HYG kin1::Kan Chk1::ura h+*	This Study
2519	*ssp2::NAT ppk9::HYG chk1::ura h+ssp2::NAT*	This Study
2520	*ppk9::HYG kin1::Kan h+*	This Study
2615	*ssp2::NAT ade6-210 h+*	This Study
2616	*ssp2::NAT ade6-210 h-*	This Study
2617	*ppk9::HYG ade6-210 h-*	This Study
2618	*ppk9::HYG ade6-210 h+*	This Study
2619	*chk1::ura ade6-210 h-*	This Study
2639	*chk1::ura ade6.210 h+*	This Study
2660	*kin1::kan ade6-210 h-*	This Study
2661	*kin1::kan ade6-210 h+*	This Study
2716	*dph1::arg rhp23::G418 h-*	This Study
2717	*dph1::arg rhp23::G418 h+*	This Study
2718	*dph1::arg3 pus1::ura h-*	This Study
2719	*dph1::arg3 pus1::ura h+*	This Study
2848	*mad2::ura ssp2::NAT*	This Study
2849	*mad2::ura kin1::G418*	This Study
2850	*mad2::ura chk1::ura*	This Study
2851	*mad2::ura ppk9::HYG*	This Study
2911	*ppk9::ppk9HA G418 3mk ade6-210 h-*	This Study
2922	*ssp2::ssp2HA G418 3mk ade 6-210 h-*	This Study
2960	*spcc1919.03c::G418 ade6.210/6.216 leu1.32 ura4.D18*	Bioneer Korea
2961	*cbs2::G418 ade6.210/6.216 leu1.32 ura4.D18*	Bioneer Korea
2962	*swi6:his1+ ade6-210 his1-102 leu1.32 uraDS/E [ch16 LEU2+ade6-216] h+*	Robin Allshire
2977	*chk1::ura ade6-210 [ch16 LEU2 ade6-216]*	This Study
2991	*ssp2::NAT ade6-210 [ch16 ade6-216 m23::LEU2]*	This Study
2992	*ppk9::HYG ade6-210 [ch16 ade6-216 m23::LEU2]*	This Study
2993	*kin1::G418 ade6-210 [ch16 ade6-216 m23::LEU2]*	This Study
2994	*spcc1919.03c::G418 ppk9::HYG*	This Study
3003	*ssp2::NAT ppk9::HYG spcc1919.03.c::G418*	This Study
3009	*slp1::slp1 GFP his+ h+*	Kevin Hardwick

2.3.2 Growth Conditions

All strains were grown at the permissive temperature of 25^{o}C on appropriate selective solid media. Temperature sensitive mutants were shifted to the restrictive temperature of 36^{o}C to induce a phenotype. Liquid cultures were seeded with 0.5-5ml of a saturated preculture and grown over night until cells were growing in log phase.

2.3.3 Storage of *S. pombe*

S. pombe was stored for short term on solid media at 4^{o}C. Stocks were stored long term at -80^{o}C in cryo tubes by adding 33.3% (v/v) YES with 40% glycerol to a saturated culture to make a final volume of 1ml.

2.3.4 Solid & Liquid Media

All liquid media made into solid media by addition of 2% (w/v) Bacto agar

2.3.5 Edinburgh Minimal Media (EMM)

0.3% (w/v) Potassium hydrogen phthalate

0.18% (w/v) Disodium hydrogen orthoposhate anhydrous

0.5% (w/v) Ammonium chloride

2% (w/v) Glucose

0.02% (w/v) of appropriate markers Adenine, Histidine, Leucine, Uracil & Lysine

2% (v/v) 50x Salts

0.1% (v/v) 1000x Vitamin

0.01% (v/v) 10,000x Minerals

2.3.6 Pombe Glutamate Medium (PMG)

0.3% (w/v) Potassium hydrogen phthalate

0.22% (w/v) Disodium hydrogen orthoposhate anhydrous

0.375% (w/v) Glutamic Acid

2% (w/v) Glucose

0.02% (w/v) of appropriate markers Adenine, Histidine, Leucine, Uracil & Lysine

2% (v/v) 50x Salts

0.1% (v/v) 1000x Vitamin

0.01% (v/v) 10,000x Minerals

2.3.7 50X Salt Stock:

5.33% (w/v) Magnesium chloride hexahdrate

0.1% (w/v) Calcium chloride hexahdrate

5% (w/v) Potassium chloride

0.2% (w/v) Disodium sulphate

2.3.8 1000x Vitamin Stock

1% (w/v) Inositol

1% (w/v) Nicotinic acid

0.1% (w/v) Calcium pantathenate

0.001% (w/v) Biotin

2.3.9 10000x Mineral Stock

0.5% (w/v) Boric acid

0.51% (w/v) Manganese sulphate tetrahydrate

0.4% (w/v) Zinc sulphate heptahydrate

0.2% (w/v) Iron (III) chloride hexahydrate

0.144% (w/v) Molybdic acid monohydrate

0.04% (w/v) Copper (II) sulphate pentahydrate

1% (w/v) Citric acid

0.01% (w/v) Potassium (I) iodide

2.3.10 Yeast Extract with Supplements (YES):

0.5% (w/v) Yeast extract (Difco)

3.0% (w/v) Glucose

0.02% (w/v) Adenine, Histidine, Leucine, Uracil & Lysine

2.3.11 Malt Extract (ME)

3% (w/v) Bacto-malt extract

2.3.12 Antibiotic Selection

Geneticin - G418 (Sigma, Ref: G-1279) and nourseothricin - NAT (Werner BioAgents Cat: 5005000) were used at 100µg/ml whilst hygromycin B - HYG (Roche; Cat: 10843555001) was used at a concentration of 300µg/ml in both liquid and solid YES/PMG but not EMM. Solid media was stored at 4^{o}C

2.3.13 Transforming *S. pombe*

20ml preculture of the strain were set up from selective plates into YES and incubated at 25^{o}C for two days. 100ml of YES was inoculated with 1-5mls of the saturated preculture and grown at 25^{o}C over night to give an OD_{600} (WPA Biowave CO8000 cell density meter, Biochrom) of between 0.5-0.8. Cultures were pelleted and washed in dH_2O then 0.1M LiAc pH4.9. Cells were resuspended in 0.1M LiAc to give a final volume of 100µl for every 0.1 OD_{600}. 1µg of plasmid was mixed with 100µl of resuspended cells along with 5mg of tRNA (Sigma) and 370µl of 50% PEG. The cells were incubated at 25^{o}C for 50mins before heat shocking in a waterbath at 46^{o}C for 20mins. After pelleting cells were resuspended in 1ml YES and incubated at 25^{o}C for 3 hrs. 200µl were plated onto selective plates and grown at 25^{o}C until single colonies were visible.

2.3.14 Converting G418 to NAT & HYG

Strains were converted from geneticin (G418/Kan) to either hygromycin B (HYG) or nourseothricin (NAT) resistance by amplifying the resistance genes in a MX6 cassette. PCRs were pooled and DNA precipated to allow 3mg of DNA to be transformed as

described previously. Cells were plated onto YES to recover overnight at 25^{o}C before being replica plated onto selective plates.

2.3.15 HA Tagging of *S. pombe* Genes

Forward primers were designed which covered the last 80bp of the gene to be tagged and the stop codon sequenced was removed. To this a 20bp sequence was added which would align to the tagging cassette used. For the reverse primer an 80bp sequence that was 200bp from the end of the gene, which did not overlap the neighbouring gene, was used with a 20bp sequence which would align to the tagging cassette at the start of the primer. These primers were then used to produce 10 PCR reactions from the tagging cassette which has a G418 resistance marked associated with it. The pooled PCRs were ethanol precipitated before being used to transform the appropriate *S. pombe* strain which were recovered over night on YES before being replica plated onto G418 plates to select for successful intergrants. These positive cells were then tested by Western blot to confirm the presence of the tagged protein.

2.3.16 Crossing Strains

Strains of opposite mating types were crossed using ME plates lacking nitrogen since starvation induces mating. 30-40µl of sterile dH_2O was pippetted onto the plate and a loop full of each yeast strain were mixed in the water before being incubated for 2-3 days at 25^{o}C. The cultures were then examined microscopically to identify successful crosses by looking for the presence of asci.

2.3.17 Tetrad Analysis

Tetrad analysis was carried out on successful crosses which were then dissected using a Singer Micromanipulator. 20µl of sterile water was pippetted onto a YES plate and a small amount of cells from the centre of the cross were mixed in it before being spread across the plate in a single line. The micromanipulator was then used to identify asci and using the fine needle asci were picked up and set out in an orderly grid. The plates were then incubated overnight at 20^{o}C to allow the ascus to burst; the four spores were moved onto separate squares of the grid in an orderly fashion. The resulting cells were then

allowed to grow before being replica plated on to two plates each containing a selectable marker, or for temperature sensitive mutants, onto YES then place at 36°C. The results were then analysed to identify double mutants.

2.3.18 Free Spore Analysis

Strains of opposite mating types were mixed together on ME plates and incubated for 2 days at 25°C as described above. For each cross 1ml of dH_2O was mixed with 5µl β-Glucuronidase (Sigma, Ref: G0876) before sterilising through a filter and aliquoted into 1.5ml eppendorfs. A loop full of the crosses taken from the middle of the cross was added to the eppendorf and incubated over night at 25°C. The remaining spores were washed twice by spinning for 1min at 13,000rpm and resuspending in 1ml of dH_2O. Six 1:10 serial dilutions of each cross were made and 100µl were plated on YES for single colonies. These were then patched and replica plated onto selective plates.

2.3.19 Spot Growth Assay

5ml of media was inoculated with a yeast colony and incubated at 25°C overnight. The cultures were then adjusted so that each culture had the same OD_{600} of 0.5-0.8. Dilutions of each culture were set up so that 1# was 1:1 dilution of the original culture, 2# was a 1:4 dilution of the original culture and 3# to 6# were 1:4 dilutions of the previous dilution. 5µl of the dilutions were spotted onto a selective media plate and allowed to dry. The plates were then incubated at 25°C to 36°C for 2-5 days.

2.3.20 Chromosome Loss Assay

Mutant strains were crossed so they contained the *ade6-210* allele to allow them to be tested. These strains were then crossed to strain carrying the mini chromosome, Ch16 which contains the allele's *ade6-216 m23::LEU2*. The two ade alleles compliment each other making the desired strain ade^+ and leu^+. The strains were tested by growing a culture to log phase in PMG-leu-ade to maintain the chromosome, then plating 200 cells (Z2 Coulter® Particle Count and Size Analyzer, Beckman Coulter™) on PMG 1/15ade. Cells were incubated at 30°C until colonies appeared then incubated at 4°C for 3 days to allow the colour to develop. Colonies with at least half their sector red were counted

along with the number `of white colonies. The percentage of chromosomes lost per division was calculated by dividing the number of red colonies by the total number of colonies and multiplied by 100.

2.3.21 MMS Sensitivity

Cultures were grown over night at 25^{o}C or 30^{o}C to log phase then 20µl of 1/1000 dilution was plated out onto two YES plates. Methyl methanesulfonate (MMS) (Sigma, Ref: M4016) was added to a final concentration of 0.005% and the cultures were grown for a further four hours; after which 20µl of 1/1000 dilution of culture was plated onto YES plates and allowed go grow to single colonies. Viability after 4hr treatment was expressed as a percentage of the colonies of the corresponding 0hr plate.

2.4 *Saccharomyces cerevisiae*

2.4.1 *S. cerevisiae* Strains

For all yeast two hybrid experiments the PJ69-4A *MATa trpl-901 leu2-3,112 ura3-52 his3-200 ga14A ga18OA LYSZ::GALl-HIS3 GAL2-ADE2 metZ::GAL7-lacZ* strain was used.

2.4.2 Solid & Liquid Media

All liquid media made into solid media by addition of 2% (w/v) Bacto agar

2.4.3 Yeast Extract-Peptone-Adenine-Dextrose (YPAD)

1% (w/v) Bacto-yeast extract

2% (w/v) Bacto-peptone

2% (w/v) Dextrose

0.01% (w/v) Adenine sulfate

Made up with dH_2O

2.4.4 Synthetic Complete (SC)

0.67% (w/v) Yeast nitrogen base without amino acids

5% (v/v) 20x Dropout Solution

2% (w/v) glucose

Made up with dH_2O adjust to pH5.8 before autoclaving

1% (v/v) of appropriate 100x Amino Acid Stock markers

2.4.5 20x Dropout

0.04% (w/v) L-Arginine HCl

0.04% (w/v) L-Methionine

0.04% (w/v) L-Uracil

0.06% (w/v) L-Isoleucine

0.06% (w/v) L-Lysine HCl

0.06% (w/v) L-Tyrosine

0.1% (w/v) L-Phenylalanine

0.3% (w/v) L-Valine

0.4% (w/v) L-Threonine

Made up with dH_2O and autoclave

2.4.6 100x Amino Acid Stock

0.2% (w/v) L-Adenine

0.2% (w/v) L-Histidine HCl Monohydrate

1.0% (w/v) L-Leucine

0.2% (w/v) L-Tryptophan

Filter sterilise

2.4.7 Yeast Two Hybrid Direct Interaction

pJ69 4A were grown in 10ml of YPAD at 30°C over night before making up to 50mls in YPAD and growing for another 2-3 hours. Cells were pelleted and washed in 40mls of TE before being resuspended in 2mls 0.1M Lithium Acetate/0.5xTE. 100µl of cells were mixed with 5µl of ssDNA and ~150ng of plasmids from the Matchmarker™ (Clontech; Cat: K1605-1) with 700µl of 0.1M lithium acetate/40%PEG/TE and incubated for 30mins at 30°C. Cells were then heat shocked at 42°C for 7min before spinning and resuspended in 200µl dH_2O and 20µl spots were plated on to SC-Leu-Trp, SC-Leu-Trp-His and SC-Leu-Trp-His-Ade. Plates were incubated at 30°C

2.4.8 Yeast Two Hybrid Screen

pJ69 4A were transformed with pGBT9 constructs containing genes to be tested and plated on SC-Trp. A positive colony was grown in 2.5ml of SC-Trp media overnight at 30°C, 180rpm before being scaled up to 50ml YPAD and grown in the same conditions for 4 hours. After centrifuging for 3 mins at 3,000rpm the cells were washed with 25ml dH_2O and resuspended in 1ml 0.1M LiAc pH 8.5 and spun for 1min at 14,000rpm and resuspended in 500µl 0.1M LiAc pH 8.5. To 200µl of cells, 100µl of DNA library was added along with 10µl ssDNA, 500µl 50% PEG and 72µl 1M LiAc pH 8.5. Cells were incubated at 30°C for 30mins before heat shocking at 42°C for 20mins, and then pelleted by spinning at 6,000rpm for 5mins. After resuspending in 1ml YPAD the cells were allowed to recover at 30°C for 45mins before being pelleted again and resuspending in 3mls of dH_2O. 100µl were plated on a small SC-LW plate whilst the remaining suspension was split evenly between 5 large SC-LWHA plates. Plates were incubated for five days at 30°C.

2.4.9 DNA Extract from *S. cerevisiae*

10ml cultures of positive colonies were grown for 30hrs at 30^{o}C and 280rpm, from which 3mls was taken and pelleted by spinning for 5min at 3000rpm. The pellet was then resuspending in 120µl of Buffer P1 from QIAprep® Spin Miniprep Kit (QIAGEN; Cat: 27106) and lysied by adding 130µl of acid washed beads and put in a Ribolyser for 10 sec at 6.5 three times with 5min incubation on ice in between. The lysate was spun for 5 mins at 14,000rpm and the supernatant was placed into a fresh tube. 120µl of Buffer P2 was added and mixed by inverting the tube before incubating for 4mins at room temperature. 180µl of Buffer P3 was added and mixed before a 5 min incubation on ice. The mixture was centrifuged at 14,000rpm for 10mins then the plasmids were isolated as described in the QIAprep® Miniprep handbook.

2.5 DNA Methods

2.5.1 Plasmids

Plasmids used in this study are shown in table below.

Name	Use	Company
pGEX KG	N Terminal GST tag	-
pGEX 6P1	N Terminal GST tag	GE Healthcare
pREP1	nmt promoter high expression in pombe	-
pREP41	nmt promoter medium expression in pombe	-
pREP81	nmt promoter low expression in pombe	-
pREP1-ccdB2	Gateway® compatable pREP1	Riken
pREP41-ccdB2	Gateway® compatable pREP41	Riken
pREP81-ccdB2	Gateway® compatable pREP81	Riken
pDONR™221	Gateway® entry vector	Invitrogen
pENTR™/ D-TOPO®	Gateway® entry vector	Invitrogen
pGEX 6P1 AH	Gateway® compatable N Terminal GST tag	Andrew Jackson
pGBT9	Binding Domains Y2H	Clonetch
pGAD424	Activation Domain Y2H	Clonetech

2.5.2 Primers

Primers design in accordance with manufacturer's instructions. All primers were produced by Sigma Genosys (Appendix E).

2.5.3 PCR

All PCR reactions were carried out on a Dyad™DNA Engine Thermal Cycler with heated lid. PCR reactions which did not require accurate transcription were performed in a final volume of 25µl containing 1 unit of Platinum Taq (Invitrogen, UK; Cat: 10966-018), 1x reaction buffer, 3µM Magnesium chloride, 0.2mM dNTP, 0.72µM primers and made up with dH_2O. DNA was then amplified using the following conditions:

Temp	Time	Cycle
95°C	4 mins	x1
95°C	1 min	x30
53°C	30 secs	
72°C	30 secs	
72°C	10 mins	x1

PCR for cloning was performed with either High Fidelity PCR Master (Roche, Ref: 12140314001) or 2x Phusion HF Master Mix (New England BioLabs, Inc; Cat: F-531L). For High Fidelity PCR Master each reaction consisted of 0.9µM primers, 50ng DNA, with sterile water to make the final reaction volume 50µl and 25µl Master Mix in this order. To amplify the DNA the following conditions were used:

Temp	Time	Cycle
94°C	2 mins	x1
94°C	10 secs	
52°C	70 secs	x10
72°C	2 mins	
94°C	15 secs	
54°C	30 secs	x20
72°C	1 min + 5 secs per cycle	
72°C	7 mins	x1

For Phusion HF Master Mix (New England BioLabs, Inc; Cat: F-531L) the following conditions were used. To a total volume of 50µl 0.36µM primers were added with 1x Master Mix and DNA. For conversion 3% DMSO was used to amplify pClone Nat-1 and pClone Hyg-1 which contained resistance in a MX6 cassette under the following conditions:

Temp	Time	Cycle
98°C	1 mins	x1
98°C	10 secs	
55°C	20 secs	x35
72°C	30 secs	
72°C	10 mins	x1

2.5.4 PCR of Bacteria Colonies

To screen transformation colonies for the correct insert the same mixture was made minus Platinum Taq. 12.5µl of this mixture was aliquoted into tubes and cells added using a pipette tip. Bacteria were lysed by one round of 94^{o}C for 10 mins. The reaction was then cooled to 54^{o}C for 10mins in which time 1 unit of Platinum Taq was added to the remaining reaction mix and 12.5µl added to each reaction before amplifying at:

Temp	Time	Cycle
92°C	3 mins	x35
48°C	45 secs	
72°C	1 min	
42°C	30 secs	
72°C	5 mins	x1

2.5.5 Site Directed Mutagenesis

Oligios were constructed in accordance to instructions of the QuikChange® Site-Directed Mutagenesis Kit (Stratagene; Cat: 200518). A 50µl reaction was set up containing 2.5ng/µl of each primer, 5-50ng of dsDNA, 0.2mM dNTP and 1x reaction buffer. To this 2.5U of PfuTurbo was added and run on a cycles devised by following manufacturers recommendations. 10U of Dpn1 was used at 37°C over night to digest the parental DNA. The remaining DNA was precipitated and transformed into DH5α.

2.5.6 Agarose Gel

To analysis DNA from PCRs and digestion a 1.5% (w/v) agarose gel was then cast with 0.5 µg/ml Ethidium Bromide and 1x TAE buffer [50x TAE 2M TRIS, 5.71% (v/v) Glacial Acetic Acid, 0.05mM EDTA pH 8.0] added. The PCR product was mixed with loading dye [20% (w/v) Ficoll 400, 100mM EDTA, Orange G to colour] were run at 100V along with a 1Kb DNA Ladder (Invitrogen; Cat: 15615-016). Photographs were taken under UV illuminator.

2.5.7 Bacteria DNA Extraction

DNA was extracted from 5ml overnight cultures of bacteria using QIAprep® Spin Miniprep Kit (QIAGEN; Cat: 27106) according to manufacturer's instructions.

2.5.8 DNA Agarose Gel Extraction

PCR products for pDONR™221 cloning and domain swap were purified after separation on agarose gel using QIAquick® Gel Extraction Kit (QIAGEN; Cat: 28704) according to manufacturer's instructions.

2.5.9 DNA Precipitation

DNA was mixed with 1/10 volume 3M sodium acetate pH5.2,then 2 volumes of 100% ethanol were added and incubated at -20^{o}C for at least one hour. The DNA was then pelleted by centrifuging at 14,000rpm for 15mins at 4^{o}C. The supernatant was removed and the pellet was washed with 200µl of ice cold 70% ethanol and centrifuged for 5mins at 14,000rpm at 4^{o}C. The remaining supernatant was removed and the pellet was dried in a 37^{o}C heat block before being resuspended in an appropriate volume of water or TE buffer.

2.5.10 Restriction Enzyme Digests

After preparing DNA using QIAquick® PCR Purification Kit (QIAGEN; Cat: 28104) DNA was cut with appropriate enzymes from either Roche or NEB, according to manufacturer's instructions. If a double digest was needed but the enzymes were incompatible, DNA was cut with one, cleaned using QIAquick® PCR Purification Kit and then digested with the second. Enzymes were deactivated before proceeding to ligation

2.5.11 Dephosphorylation

When only a single digest was possible vectors were dephosphorylated using either Antarctic Phosphatase (NEB; Cat: M0289S) or Calf Intestinal Alkaline Phosphatase (Invitrogen; Cat: 18009-019) according to manufacturer's instructions.

2.5.12 Ligations

Ligations were carried out using Quick Ligation™ Kit (NEB, Cat: M2200L) according to manufacturer's instructions.

2.5.13 Sequencing

Sequencing was carried out in house by MRC HGU technical Services before being loaded into an Applied Biosystems DNA sequencer (Model 373A or 377) and operated in according to manufacturer's instructions.

Sequencing data was analysed with ClustalW2 alignment tool. (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

2.5.14 Gateway® Cloning

To construct the entry vector pENTR™ TOPO® (Invitrogen, Cat: 2435-20) or pDONR™ 221 (Cat: 12536-017**)** primers were designed in accordance with Invitrogen instructions. For pENTR™ TOPO® 1-5ng of PCR product was mixed with 1µl salt solution, 15ng of pENTR™ TOPO® and made up to 5µl with sterile water and incubated at 25^{o}C between 5mins are over night. The reaction was then transformed into Transforming One Shot® Mach1™-T1® Competent *E.coli* (Invitrogen) and the remaining reaction was stored at -20^{o}C for up to a week.

For pDONR221™ PCR products were gel purified and 150ng of PCR and vector were incubates at 25^{o}C over night with 2µl of BP Clonase™ II and made up to 8µl in TE. Reactions were stopped by adding 1µl of proteinase K and incubating at 37^{o}C for 10 mins. Ligations were then transformed into DH5α and selected for on Kan plates. Both plasmids were sequenced with M13 primers.

2.5.15 Gateway® Switching to Destination Vector

To move the cloned genes from the enter vector to the destination vector, a similar reaction was set up as stated for pDONR221™ but LR Clonase™ II was used, and the resulting transformants were screened on Amp plates.

2.6 Protein Methods

2.6.1 Protein Induction

10ml L-broth precultures containing 100µg/ml ampicillin were inoculated with a single colony of BL21 contain the pGEX 6P1 plasmid and incubated over night at 37^{o}C. The cultures were added to 200mls of L-broth containing ampicillin and incubated at 37^{o}C until the culture had reached an OD_{600} of 0.4-0.8. IPTG was added to give a concentration of 0.1mg/ml and incubated at either 25^{o}C or 37^{o}C for 4 hours. Cultures were pelleted by spinning at 12,000rpm for 5mins and removing the supernatant. Pelleted cells were kept at -80^{o}C overnight before undergoing GST bead purification.

2.6.2 Late Log Phase Protein Induction

For GST-Slp1, the method described by Galloway et al. (2003) was employed. Briefly, BL21 contain the pGEX 6P1 plasmid was grown over night at 37^{o}C. The cultures were added to 100mls of L-broth containing ampicillin and incubated at 37^{o}C until the culture had reached an OD of ~1.7. Cultures were placed on iced water for 5mins then IPTG was added to give a concentration of 0.1mg/ml and incubated at 25^{o}C for 1 hour. Cultures were pelleted by spinning at 12,000rpm for 5mins and removing the supernatant.

2.6.3 GST Bead Purification

Bacterial pellets from protein inductions were resuspended in freshly made cold GST Binding Buffer [50mM TRIS pH7.5, 100mM NaCl, 10% glycerol, 1% triton and 1 Complete Tab (Roche, Ref:11697498001) per 50ml] and sonicated twice for 45secs on ice. After rotating for 30mins at 4^{o}C debris was spun down at 12,000rpm for 30mins at 4^{o}C. The supernatant was transferred into a 50ml Falcon™ tube with 200µl Glutathione Sepharose™ 4B beads (GE Healthcare, Ref: 17075601) and rotated for 1hr at 4^{o}C. The beads were then spun down at 3000rpm for 3mins and transferred to a fresh eppendorf. To wash the beads 1ml of GST binding buffer was added and inverted before spinning at 8,000rpm, this was done a total of 3 times. Induced proteins were then separated on

SDS-PAGE gels and visualised using Coomassie Blue to validate the protein was of the correct size.

2.6.4 Eluting GST Tagged Protein

To removed GST tagged protein from Glutathione Sepharose™ 4B beads, the beads were packed into a polyprep column (Biorad, Cat: 731-1550). 5mls of 100mM L-Glutathione Reduced (Sigma, Ref: G6529) in 50mM TRIS pH8 was added and collected in 1ml fractions. The samples were then analysed by separating on a 10% SDS PAGE gel and stained to see which fraction contained the eluted protein.

2.6.5 SDS-PAGE Gels

SDS-PAGE gels were cast as follows:

	10% Separating	12% Separating	Stacking
29:1 30% Acrylamide	10% (v/v)	12% (v/v)	4% (v/v)
4x Buffer	25% (v/v)	25% (v/v)	25% (v/v)
APS	0.001% (w/v)	0.001% (w/v)	0.001% (w/v)
TEMED	0.1% (v/v)	0.1% (v/v)	0.1% (v/v)

4x Separating Buffer	4x Stacking Buffer
18.15% TRIS (w/v)	6.05% TRIS (w/v)
0.4% SDS (v/v)	0.4% SDS (v/v)
pH 8.8	pH 6.8

Samples were mixed 1:1 with loading dye [2% (w/v) SDS, 62mM TRIS pH7.5, 10% (v/v) glycerol and 0.0125% (w/v) bromophenol blue] was boiled with 5% (v/v) β mercaptoethanol and loaded onto the SDS-PAGE gel with Broad Range Marker (New England Biolabs; Ref: P7708S). Gels were run in 1x Running Buffer [5x Running Buffer 1.25M Glycine, 125mM TRIS and 0.5% (v/v) SDS] at 160Volts for 1hr and either used for Western blots (See section) or stained for protein

2.6.6 Visualisation of Protein on SDS PAGE Gels

SDS PAGE gels were stained either with GelCode® Blue Stain Reagent (Pierce; Ref: 24592) according to manufacturer's instructions, or with Coomassie stain [40% (v/v) methanol, 10% (v/v) acetic acid, 0.1% (v/v) Coomassie blue] and then destained [25% (v/v) methanol, 7.5% (v/v) acetic acids].

For visualisation of proteins at a level too low to be detected by either of these methods, such as Tap Tag purified APC/C, gels were silver stained using GelCode® SilverSNAP® Stain Kit II (Pierce, Cat: 24612).

2.6.7 Transfer of Western Blots

Proteins were transferred via wet blotting using the mini trans-blot cell (Biorad). The cassettes were loaded with one pad followed by two layers of Whatman 3MM paper, the SDS-PAGE gel, a sheet of Hybond-P membrane (GE Healthcare, Ref: RPN303F) soaked in methanol, another two layers of Whatman paper and another pad all pre soaked in transfer buffer [24mM TRIS, 186.5mM Glycine, 20% (v/v) methanol]. Transfers were performed at 200mA for 1hr 30 mins, changing the ice block half way through or were blotted over night at 40mA.

2.6.8 Probing Western Blots

Membranes were blocked overnight at 4^{o}C or for 1hr at room temperature (RT) for 1hour in 5% Sainsbury's dried skimmed milk in PBS with 0.1% Tween-20 (Sigma) or 5% BSA (Melford, Cat: A1302) in TBS with 0.1% Tween-20. The membrane was probed with primary antibody in 5% milk/BSA made in PBST/TBST for 1hr then washed for three times for 10mins in PBST/TBST whilst being shaken. The secondary horse radish peroxidise (HRP) antibody was added in 5% milk/BSA in PBST/TBST for 30mins then washed three times for 10mins in PBST/TBST. The membrane was then drained on tissue and the proteins were visualised using ECL Western Blotting Detection System (GE Healthcare; Ref: RPN2132) according to manufacturer's instructions. The membranes were the exposed to Hyperfilm™ ELC (GE Healthcare) in a dark room for 5sec-30min depending on antibody.

2.6.9 Antibodies

Antibody	Concentration	Block	Source
Anti HA7	1:5000	5% Milk	Sigma
Anti Ubiquitin	1:1000	5% BSA	DakoCytomation
Anti TATA	1:20,000	5% Milk	In House
Anti GFP	1:1000	Dilution Buffer	
Anti HA	1:5000	5% Milk	
Anti Mouse Horse Readish Peroxidase (HRP)	1:5000	5% Milk or BSA	GE Healthcare
Anti Rabbit HRP	1:5000	5% Milk or BSA	GE Healthcare
Anti GST HRP	1:100,000	Dilution Buffer or 5% BSA	Pierce

2.6.10 Blot stripping

Membranes were placed in an airtight container with 100ml of stripping buffer [62.5M Tris pH8.6, 2% (v/v) SDS, 100mM β mercaptoethanol] and heated to 50^{o}C in a waterbath for 40mins. Blots were then washed six times for 5mins with PBST before re-blocking.

2.6.11 Whole Cell Protein Extract from *S. pombe*

100ml of culture was grown overnight at 25^{o}C to an O.D$_{600}$ of 0.5-1.0. Cells were pelleted in two 50ml Falcon™ tubes by centrifugation at 3,000rpm for 3mins. After two washes in dH$_2$O cells were washed in 1ml lysis buffer [50mM TRIS pH 7.5, 50-100mM NaCl, 10% glycerol, 1% triton, 1mM PMSF, 1 complete per 50ml]. The pellets were then resuspened in 200µl lysis buffer and 200µl acid washed beads. A ribolyser was used to disrupt the cells by 10secs at 6.5 with 2 mins incubation on ice three times. A further 200µl lysis buffer was added for a fourth round. Beads and debris was removed by spinning for 5mins at 8,000rpm at 4^{o}C. The supernatant was transferred to a fresh tube then spun at 14,000rpm for 30mins at 4^{o}C. The clarified supernatant was then pooled to make a whole cell extract.

2.6.12 Native PAGE

To run a native gel for far Westerns, *S. pombe* extracts were made as stated in 2.6.11, without detergents. Samples were prepared by adding a equal volume of Novex® Tris-Glycine Native Sample Buffer (Invitrogen, Cat: LC2673) and 50ng-100ng of protein were run on NuPAGE® Novec® Tris Acetate 3-8% gels (Invitrogen, Cat: EA0375BOX) in ice cold Tris Glycine Native Running Buffer (Invitrogen, Cat: LC2672) at 150V for 3 hours whilst keeping the unit as cold as possible.

2.6.13 Transfer of Native PAGE

Proteins were transferred via semi dry blotting using the MilliBlot™ Graphite Electroblotter II (Millipore, Cat: MBBDGE002). Two layers of Whatman 3MM paper with a sheet of Hybond-P membrane soaked in methanol, the SDS-PAGE gel and another two layers of Whatman paper all pre soaked in 2xTris Glycine Transfer Buffer (Invitrogen, Cat: LC3675) were layered onto the blotter and run at 100mAmps per blot for 1hour.

2.6.14 Probing Native Far Western

Far western were performed using the ProFound Far-Western GST-Protein:Protein Interaction Kit (Pierce, Cat: 23505) according to manufacturer's instructions. Briefly, membranes were blocked over night at 4^{o}C in dilution buffer or 5% BSA. 1-5ng/ml of eluted GST protein was added to dilution buffer, or dilution buffer on its own was added as a negative control. To probe tagged protein, the appropriate antibody was added. Membranes were incubated for 1 hour at room temperature with shaking then washed 3 times for 15mins in PBST. αGST- HRP antibody was added to test and negative control membranes in dilution buffer whilst the appropriate secondary antibody was added to the tag control. After one hour membranes were washed 3 times for 15mins in PBST. Blots were then visualised with UniBlot provided and exposed to x-ray film.

2.6.15 APC/C Interaction Assay

The strain c*ut9::cut9-3HALeu2+(leu1.32)h*$^{-}$ was used since there are no antibodies that can accurately detect APC/C. This strain has a C-terminal HA tag *cut9* under control of

its native promoter. These cells are viable and since *cut9* is an essential gene it can be assumed that the HA tag does not affect Cut9s function.

200mls of YES was inoculated with 1-3mls of a 20ml preculture and grown overnight at 25^{o}C to give an OD_{600} of 0.8-1.0. The cultures were pelleted at 3000rpm for 3mins at 4^{o}C and washed twice in cold dH_2O. After resuspending in 1ml of lysis buffer [50mM TRIS pH7.5, 100mM NaCl, 10% glycerol, 1% triton, 1mM PMSF, 1mM DDT, 2mM ATP and 1 Complete Tab per 50mls] cells were transferred to screw capped tubes and spun at 8000rpm for 3min before being suspended in 200µl lysis buffer. 200µl of ice-cold acid washed glass beads were added to each tube before undergoing three rounds of lysis in the Fastprep for 10sec at 6.5 followed by 2min incubation on ice. A further 200µl of lysis buffer was added before one final round of lysis. The preparations was then spun for 5min at 8000rpm and the supernatant transferred to a fresh tube then spun twice for 30min at 13000rpm and the supernatant collected.

Protein concentration was measured on a NanoDrop ND-1000 Spectrophotometer set at an absorbance of 280nm and adjusted to give 50mg/ml. 20µl of the extract was mixed with 20µl of 2xSDS loading buffer as the input sample. GST tagged proteins bound to GST beads were washed twice in lysis buffer and 5-30µl of the beads were rotated overnight at 4^{o}C with 100µl of protein extract. Beads were then washed 5 times with freshly made lysis buffer before being prepared for Western blots.

2.6.16 TEV Purification

400ml of 6xHis TEV amp resistant BL21 (DE, no pLysS) was grown to an OD of 1 and induced for 4 hours at 37^{o}C with 20mg of IPTG. The pellet was weighed and resuspended in 4 volumes per gram of cell in lysis buffer [20mM TRIS Cl pH8, 1mM EDTA, 8U/ml DNase I, 400µg/ml lysozyme, 1 complete tablet/50ml] and incubated at room temperature for 20mins. The cells were lysed by sonication three times for 45 secs with 1min incubation on ice in between. After another 5min incubation at room temperature Triton-100 was added to 1% concentration and NaCl to 300mM. Cells were pelletted by centrufication at 10,000rpm for 20mins at 10^{o}C. The pellet was resuspended

using a homogeniser in 8-10 volumes per gram of cell resuspension buffer [50mM TRIS pH8, 300mM NaCl, 1% Triton-100, 1 complete tablet/50ml], then centrifuged. This process was repeated before the pellet was resuspended in 4 volumes per gram of cell 6M guanidine HCl 3% buffer B [25mM TRIS pH8, 300mM NaCl] 97% buffer A [25mM TRIS pH8, 300mM NaCl, 300mM Imidazole] using a homogeniser. The suspension was heated to 65^{o}C for 20mins and an equal volume of 3%B/97%A was added. After spinning at 15,000rpm for 20mins at 10^{o}C the supernatant was collected and placed onto 5ml Ni-NTA agarose pack in a column equibrilated with 2 column volumes of 3%B/97%A. The column was washed 4 times with column volume of 8M urea 3%B/97%A then 4 times with column volume of 8M urea 10%B/90%A. To elute the columns were washed 4 times with column volume of 8M urea 30%B/70%A then 4 times with column volume of 8M urea 100%B. The first 33% of the columns volume from the elute, which is the void volume, was discarded and the TEV proteins was collected in 4 fractions of column volume. A 10% SDS PAGE gel was run and stained with Coomassie to confirm with fractions contain the TEV and to check the purity. These fractions were pooled and put through a PD-10 buffer exchange column (Pharmacia Biotech; Code No: 17-0851-01) to transfer the TEV into Dialysis Buffer [100mM TRIS pH 8.5, 0.5M NaCl, 5mM DTT, 0.5mM EDTA, 50% glycerol]. TEV activity was tested and aliquots were stored at -80^{o}C

2.6.17 TAP Tag Purification

To 50ml of NP-40 buffer [6mM Na_2HPO_4, 4mM $NaH_2PO_4{\cdot}H_2O$, 1% NP-40, 150mM NaCl, 2mM EDTA, 50mM NaF] 1 complete EDTA free, 1.3mM benzamidine and 1mM PMSF was added fresh before use. Pellets were resuspended in 1ml of this buffer and cells were disrupted by adding 200µl of acid washed glass bead to 200µl of the cell suspension and ribolysed using a Hybaid RiboLyser at a power of 6.5 for 10second. This was repeated three times with 2 min incubation on ice in between. A further 100µl of buffer was added before a fourth round in the ribolyser. Debris was then removed by centrifugation at 8,000rpm for 5mins at 4^{o}C, whilst the supernatant was pooled and

incubated with 200µl of IgG Sepharose 6 Fast Flow beads (GE Healthcare, Cat:17-0969-01) which had been prequilibrated with NP-40 buffer overnight at 4°C.

The lysate and the beads were then transferred to a polyprep column and the lysate was drained off. Beads were then washed with 10ml of IPP150 [10mM TRIS-HCL pH8.0, 150mM NaCl, 0.1% NP-40] followed by 5ml of TEV cleavage buffer (TEV CB) [10mM TRIS-HCL pH8.0, 150mM NaCl, 0.1% NP-40, o.5mM EDTA, 1.0mM DTT]. The proteins were then cleaved off the IgG beads by addition of 1ml of TEV CB and 100U of TEV overnight at 4°C. The proteins were then eluted into a fresh column and the beads washed with 0.5ml TEV CB. Three volumes of IPP150 Calmodulin binding buffer (CBB) [10mM TRIS-HCL pH8.0, 150mM NaCl, 1mM Mg^{2+} acetate, 1mM Imidazole, 2mM $CaCl_2$, 10mM βME, 0.1% or0.02% NP-40] was added to the elute along with 3µl of 1M $CaCl_2$ per 1ml of elute.

Protein was then bound to Calmodulin Affinity Resin (Stratagene, Cat: 214303-52) prequilibrated with CBB overnight at 4°C. Beads were washed twice with 1ml CBB 0.1% NP-40, once with 1ml CBB 0.02% NP-40 before the final purified protein was eluted with 500µl of IPP150 Calmodulin elution buffer (CEB) [10mM TRIS-HCL pH8.0, 150mM NaCl, 0.02% NP-40, 1mM Mg^{2+} acetate, 1mM Imidazole, 20mM EGTA, 10mM βME]

2.6.18 Ubiquitin Binding Assay

To 50ml TBS [12.5mM TRIS pH7.5, 37.5mM NaCl] one complete – EDTA tablet was dissolved. Either K48 tetra ubiquitin (Enzo life science, Cat: UW8645) or K48 chains (Enzo life science, Cat: UW8860 or Boston Biochem, Cat: UC-230) or K63 chains (Boston Biochem, Cat: UC-320) was made up to a concentration of 6.25mg/l or 12.5mg/l. 100µl of the ubiquitin chains were added to 15-30µl of GST tagged proteins bound to Glutathione Sepharose™ 4B beads, or 2-5µl for GST alone. Samples were rotate for at least 2 hrs or overnight at 4°C before being washed 5 times by inverting tube with 1ml of TBS.

The protein was the boiled off the beads using SDS PAGE loading buffer and an equal amount of GST tagged protein was loaded onto two 10% SDS PAGE gels. One was strained to show proteins loading whilst the other was blotted for use in Western analysis. For the Western analysis, membranes were blocked in 5% BSA in TBST.

Chapter 3
Interaction of KA1 and KA2 Domains with APC/C

3.1 Introduction

APC/C is a RING type E3 ubiquitin ligase which is part of the regulation process in the cell cycle. One of the ligases most important roles is to mark crucial proteins for destruction. These proteins include cyclins, which are involved in each stage of the cell cycle and the cohesion complex, which hold the sister chromatids together before entering anaphase (Jallepalli et al. 2001; Bastians et al. 1999). In fission yeast APC/C recognises the protein Cut2 (commonly called securin), an inhibitor of the enzyme Cut1 (separase). When separase is released from its inhibition, it cleaves the Rad21 (Scc1) subunit of the cohesin complex, allowing the sister chromatids to separate and move to opposite poles of the dividing cell (Chang et al. 2001).

The activity of E3 ligases are regulated in two ways. Some ligases are always in an active state e.g. SCF, and depend on the phosphorylation state of the substrate as a trigger to recognise their targets. Where substrate recognition is predetermined the ligase activity is regulated, as in the case of APC/C.

APC/C always recognises its substrates through two co-activators, Slp1 (commonly known as Cdc20) and Ste9/Srw1 (Cdh1) (Acquaviva & Pines 2006) but is tightly regulated to dictate when the ligase is activated to ubiquitinate its substrates. In higher eukaryotes APC/C is a target for kinases such as PKA and PLK1 (Acquaviva & Pines 2006). The resulting phosphorylation can either positively or negatively affect the ligase activity. Another form of regulation is provided by the Mitotic Checkpoint Complex (MCC) which inhibits APC/C^{Slp1} by binding to the co-activator, possible preventing APC/C from binding its substrates (Chung & Chen 2003; Burton & Solomon 2007). These layers of regulation along with the two co-activators; Slp1, which is active from metaphase to anaphase and Ste9, which is active from anaphase to telophase, allow for APC/C to be active and to recognise the correct substrate at the precise time, preventing cells from becoming aneuploidy (Engelbert et al. 2008).

A study by Yamada et al. (1997) used an *S. pombe* strain in which endogenous *cut9+* had been replaced with a HA epitope tagged *cut9+* gene under the control of the *cut9+* promoter. They showed by Western blot analysis that APC/C from this strain contains two forms of Cut9-HA corresponding to a higher molecular weight modified form and a lower molecular weight unmodified form. Protein extracted from synchronised cultures demonstrated that during most of the cell cycle APC/C contained the lower molecular weight Cut9 except through metaphase when Cut9 appears modified, and suggested that this modification was important to APC/C activity. APC/C was immunoprecipitated from extracts using anti-HA antibodies and treated with alkaline phosphatase, which caused the higher molecular weight Cut9 species to disappear, and alkaline phosphatase plus an inhibitor which maintained the higher weight Cut9 proving this modification was due to phosphorylation (Yamada et al. 1997). Ongoing studies within this group have shown that two related fission yeast kinases, Ssp2 and Ppk9, are capable of binding APC/C containing just the unphosphorylated form of Cut9-HA. To determine if both proteins share a common domain which could be responsible for this interaction, the protein sequences were sent for bioinformatics analysis to Kay Hofmann (Miltenyi Biotec GmbH, Stoeckheimer). He concluded that both kinases contain a novel Kinase Associated 1 (KA1) domain at their C-terminus. This domain is also present in another *S. pombe* kinase called Kin1 (Tassan & Le Goff 2004). In attempts to identify other kinases which may contain a KA1 domain, it was revealed that a very similar domain existed at the C-terminus of the kinase Chk1 which has now been designated as the Kinases Associated 2 (KA2) domain.

The KA containing kinases belong to three different groups. The first group include Ssp2 and Ppk9, which are alternative catalytic α-subunits of *S. pombe* AMPK, a multimeric enzyme that in other organisms is involved in energy homeostasis (Matsusaka et al. 1995; Hanyu et al. 2009). However, very little work has been carried out on these kinases in fission yeast. During times of glucose limitation, AMPK is activated and drives the transcription of glucose repressible genes which allow the cell to compensate for these low levels of glucose.

The second group is the MARK family of kinases, where Kin1 is the only *S. pombe* homologue. These kinases are responsible for governing cell polarity and were the first family of proteins to have a defined KA1 domain at their C-terminus (Levin & Bishop 1990). The primary role of these kinases is to phosphorylate MAP proteins. MAP proteins bind to microtubules and stabilise them using a domain that is also a target for phosphorylation by MARKs. This phosphorylation event causes the MAP proteins to dissociate from the microtubules which in turn become less stable (Trinczek et al. 1995; Drewes et al. 1997; Ebneth et al. 1999).

The third group is the KA2 containing Chk1 kinase, which is involved in the DNA damage response cascade (Walworth et al. 1993). When Chk1 is activated it inhibits the dephosphorylation of the Cdc2 (Cdk1) and as a result the Cdc2-Cdc13 (CyclinB) complex remains inactive, causing a delay in the G_2/M phase transition giving the cell time to repair any DNA damage (Karlsson-Rosenthal & Millar 2006).

The MARK family of kinases are found in many organisms from yeast and *Caenorhabditis elegans* up to humans. They are identified as containing an ~50 amino acid KA1 domain which previously was defined by i) a conserved aspartic acid residue and ii) a C-terminal Glu-Leu-Lys-Leu (ELKL) motif at the end of the protein (Tassan & Le Goff 2004). However, this original definition has been modified as more KA1 domains have been discovered, some from their sequence others by their structure. Several KA1 domains are up to 100 amino acids in length and it is believed that the extended sequence is important for the domain structure. Similarly KA1 domains such as the ones found in Ssp2 and Ppk9 lack the conserved ELKL C-terminal motif found in other KA1 domains, which had also been thought to play an important role in the domains structural fold. Surprisingly others do not contain a conserved aspartic acid, although some have a conserved glutamic acid (Tochio et al. 2006; Townley & Shapiro 2007; Sanger 2010).

This chapter confirms the interaction of these four kinases with APC/C *in vitro* by using the *S. pombe cut9-HA* tagged strain. Results show that the KA domain is responsible for

the observed interaction with APC/C and unlike the shuttle factors Pus1 and Rhp23, the KA domain specifically recognises APC/C containing the unphosphorylated form of Cut9. To identify which subunit of APC/C is responsible for the interaction with the KA domain, the yeast two hybrid protein/protein interaction assay was used. Unexpectedly, the data demonstrated that the KA domains do not interact directly with any of APC/Cs core subunits. A yeast two hybrid screen suggested that the KA domains may, in fact, bind to the co-activator Slp1. In order to confirm this interaction; native far Westerns were performed using GST tagged KA domains which confirmed that the KA domains bound to GFP tagged Slp1.

3.2 KA Domains Bind Specifically to APC/C with Unphosphorylated Cut9

Studies by Yamada et al. (1997) showed that using whole cell extracts from a *cut9-HA* strain, two bands of different molecular weight were present when analysed by Western blots. They demonstrated that the higher molecular weight band was due to phosphorylation of Cut9 whilst the lower molecular weight protein was unphosphorylated Cut9.

In order to determine if the KA domain was responsible for the binding of the kinases to APC/C, full length and deleted version of the kinases were cloned into the Gateway® entry vectors pDONR™221 and pENTR™ D-TOPO®. The three versions of the proteins cloned were: i) full length kinase, ii) KA domains only which were taken to be residues: 445-576 for Ssp2, 421-532 for Ppk9, 751-891 for Kin1 and 393-496 for Chk1 and iii) the full length kinases with the KA domain deleted (ΔKA). Although *chk1+* full length DNA could be amplified by PCR no entry vector containing a WT full length *chk1+* could be constructed. These were then transferred into Gateway® modified pGEX 6P1 AH vector (gift from Andrew Jackson) to express GST tagged proteins in BL21strain of *E.coli* and purified onto Glutathione Sepharose™ 4B beads. The protein coupled beads were then incubated with extracts from *cut9::cut9HA* strains and washed

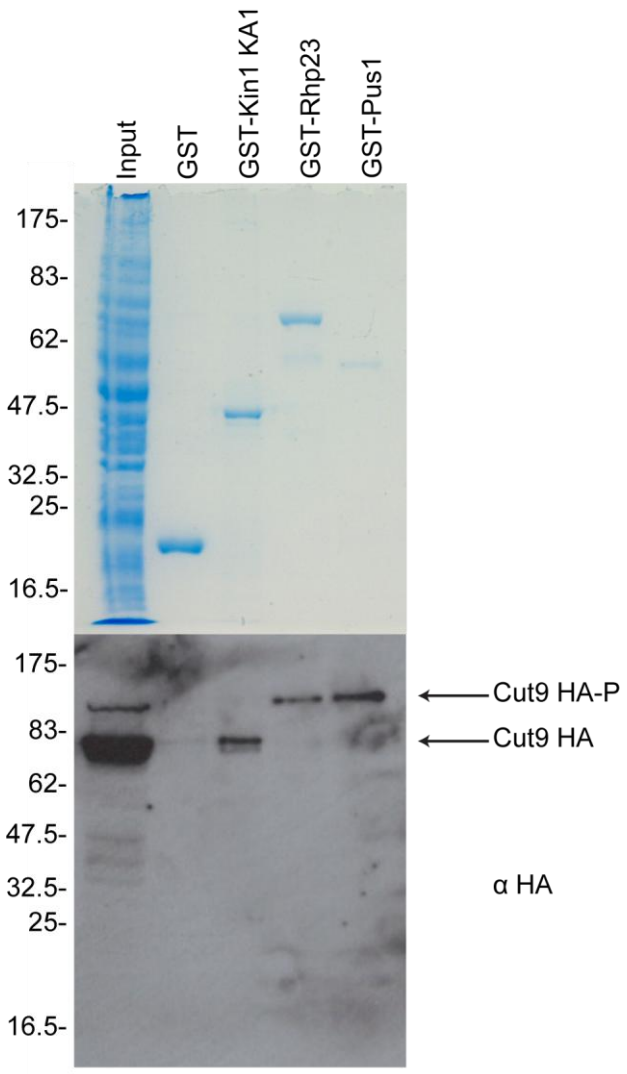


Figure 10: Kin1 KA1 Domain Binds to Unphosphorylated Cut9 Form of APC/C.

GST fusion proteins were expressed in bacteria and purified onto glutathione beads. Next they were incubated with whole cell lysate from a strain carrying a HA tagged form of Cut9 before unbound protein was removed by washing. Top panel showing a Coomassie stained SDS PAGE gel of the input GST tagged protein. The bottom panel shows the corresponding Western blot for Cut9-HA probed with anti-HA antibody (α HA). Protein extracts from the *cut9-HA* tagged strain (input) produces two bands corresponding to phosphorylated and unphosphorylated protein. The GST control did not bind APC/C whilst the shuttle proteins, Rhp23 and Pus1 can bind APC/C containing the phosphorylated form of Cut9. The KA1 domain of Kin1 binds the unphosphorylated form of APC/C exclusively.

to remove unbound protein. The GST fusion proteins were separated by 10% SDS PAGE and Coomassie stained to show equal loading of the input GST protein. Western blot analysis was carried out on the samples probing with anti HA antibody (αHA) to detect APC/C binding by the presence of the Cut9-HA protein.

To determine if the KA1 domain was responsible for the interaction with APC/C, the KA1 domain of Kin1 was tested since it appeared to be the best defined and it is the only KA domain, from the four kinases, which contains the ELKL motif. The KA1 domain of Kin1 is predicted to be 15.6kDa and Coomassie stained gel showed that the expressed GST tagged protein runs at just under 47.5kDa (Figure 10) which is consistent with the predicted size of the tagged domain. The Western blot of the APC/C binding assay concluded that GST alone could not bind to APC/C and that just the KA1 domain of Kin1 was sufficient to bind APC/C. This experiment also confirmed that the KA1 domain only recognises APC/C containing unphosphorylated Cut9, compared with the shuttle factors Rhp23 and Pus1 which only recognised APC/C containing phosphorylated Cut9. The full length proteins of Ssp2, Ppk9 and Kin1 were then tested for their APC/C binding ability using the same assay (Figure 11 **a**).

All three full length proteins bound to APC/C containing unphosphorylated Cut9. The same result was produced when only the KA domain of kinases was used (Figure 11 **b**). Although not quantitative, another observation from this data was that the Kin1 KA domain bound APC/C better than the other three domains. Ssp2 full length, ΔKA1 and KA1 were tested to confirm that the KA domain was responsible for the interaction. Both full length and KA1 domain of Ssp2 bound APC/C, but the ΔKA1 protein could not (Figure 11 **c**), supporting the theory that only the KA domain is responsible for the observed interaction.

Sequence comparisons predict that Ssp2 has a putative UBA domain. It is known that kinases in Kin1 family of kinases also possess UBA domains. The interaction of the shuttle factor Rhp23 with APC/C is reliant on its UBA domains and it has been suggested that the UBA is binding ubiquitinated substrates attached to APC/C

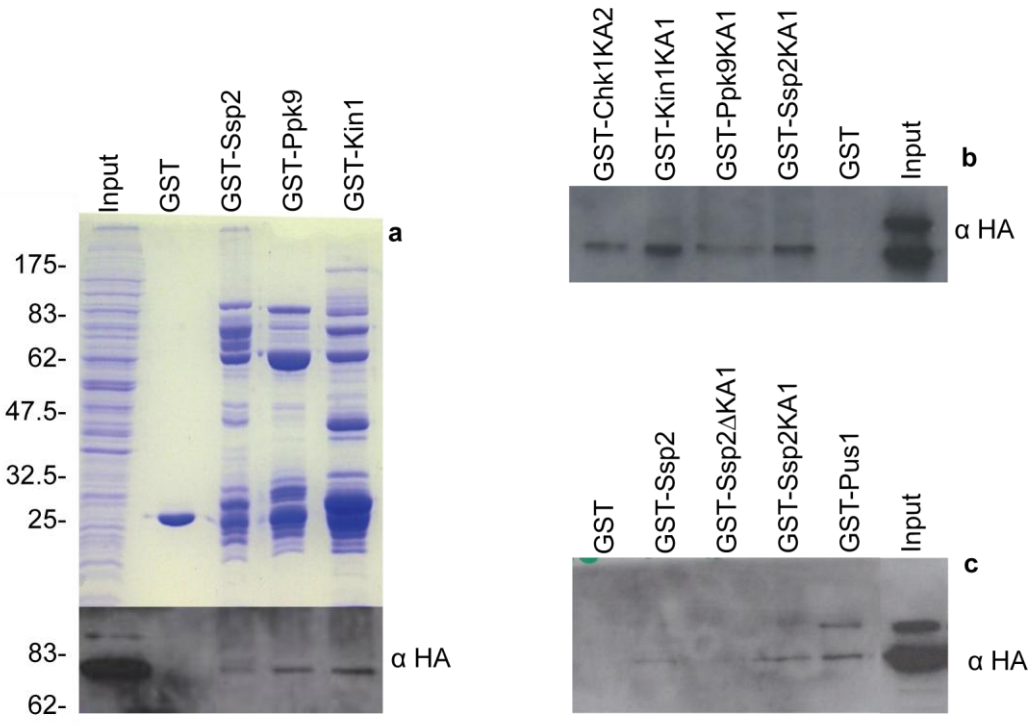


Figure 11: Full Length Protein and KA Domains Binds to Unphosphorylated Cut9 Form of APC/C. GST fusion proteins were expressed in bacteria and purified onto glutathione beads. They were then incubated with whole cell lysate from a strain carrying a HA tagged form of Cut9 before unbound protein was removed by washing. **a)** GST tagged full length protein for Ssp2, Ppk9 and Kin1 (top panel Coomassie stained) were capable of binding APC/C containing unphosphorylated Cut9 (bottom panel α HA blot). **b**) The same result was observed when all four isolated KA domains were tested for the ability to interact with APC/C. **c**) Full length, ΔKA1 and KA1 proteins of Ssp2 was expressed in bacteria and tested for their ability to bind APC/C to confirm no other region of the protein was responsible for binding. Full length and the KA1 domain of Ssp2 could bind APC/C, however Ssp2 ΔKA1was unable to interact with APC/C.

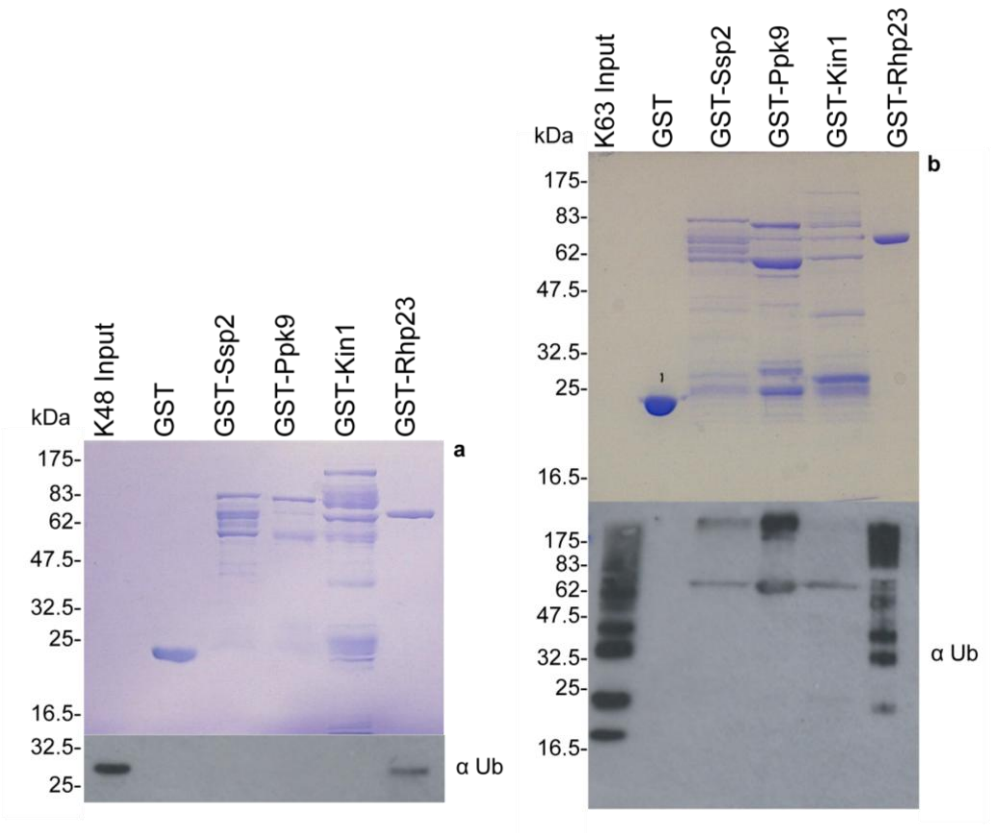


Figure 12: Full Length Proteins Do Not Bind Ubiquitin Chains.

GST tagged full length proteins were expressed in bacteria and coupled onto beads. After incubation with K48 (**a**) or K63 (**b**) ubiquitin chains, the beads were washed and separated on 10% SDS PAGE to stain with Coomassie (top panels) and analysed by Western blot for their ability to bind ubiquitin chains (bottom panels). None of the three full length proteins tested could bind ubiquitin chains unlike Rhp23, confirming that any bind to the APC/C complex is not via ubiquitin chains, and suggesting that the UBA domain of Ssp2 is has no ubiquitin binding activity.

(Seeger et al. 2003). To rule out this possibility the three full length proteins were tested for their interaction with K48 and K63 chains (Figure 12). These results showed that none of the proteins could bind ubiquitin chains; therefore the interaction with APC/C is not due to ubiquitin recognition.

3.3 The KA Domains Do Not Bind Directly to a Core APC/C Subunit

Although the binding assays confirmed that the KA domains bind APC/C and specifically APC/C containing unphosphorylated Cut9, the actual subunit of APC/C involved in this interaction has not been determined. Using the direct yeast two hybrid assay system, interactions between the four KA domains and the APC/C subunits could be observed. In this assay the strain J69-4A (James et al. 1996) was used which contains in its genome three binding sites: GAL1, GAL2 and GAL7 which are upstream of the selective markers: HIS3, ADE2 and lacZ respectively. Bait proteins were produced by cloning genes into the pGBT9 vector which encodes a binding domain for the above promoters. Prey constructs were produced by the pGAD424 vector which encodes a transcription activation domain. When the fusion proteins are translated the binding domain recognises the binding site and holds the bait protein in close proximity to the selective gene. If the prey protein interacts with the bait, it will allow the activation domain to transcribe the selectable marker which leads to a visible read out.

The KA domains and APC/C subunits were cloned into the pGBT9 binding domain vector and pGAD424 activation domain vector. However the two APC/C subunits *apc2+* and *apc15+* could not be cloned into either vector so could not analysed. Initially, the ability of the different subunits to self activate was tested. To do this the vector containing the test gene and the opposite empty vector were transformed into PJ69-4A and spotted onto selective media lacking leucine and tryptophan (SC-LW) to test for successful transformation; media lacking leucine, tryptophan and histidine (SC-LWH) to test for weak interaction and media lacking leucine, tryptophan, histidine and alanine (SC-LWHA) to test for strong interaction. None of the KA domains self

activated or interacted with each other (data not shown). pGBT9 *apc13+* showed self activation when tested with empty pGAD424 however the reverse experiment with pGAD424 *apc13+* did not. All four KA domains were tested against APC/C subunits which did not self activate along with SNF1 and SNF4 as a positive control (See Appendix A). Unexpectedly none of the subunits showed interaction with the different KA domains. Proteins that make up the MCC were also tested since this complex binds to APC/C and regulates its activity. The four KA domains did not interact with Mad2, Mad3 or Bub3 (Table 2).

As an alternative approach to identify proteins which interact with the KA domains, the pGBT9 constructs were used as bait for a yeast two hybrid screen using the Beach pACT cDNA library. J69-4A cells were transformed with a KA containing pGBT9 first, then cells containing the binding domains vector were transformed with the activation domain library to maximise efficiency and then screened on SC- LWHA to find strong interactors. These positive colonies were then re-tested before being cultured and their DNA extracted. Activation domain primers were used to perform PCR from these DNA sources and then only the single band products were sequenced to identify the interactants (Table 3).

The largest number of interactors was seen with Chk1 KA2 which gave 81 colonies. Of these 66 appeared to be a result of a colony containing a singly transformed pACT construct which gave good sequence. 42 encoded ribosomal proteins, the most common being *rps002* which was responsible for 32 positive hits. Ribosomal proteins are common interactors in yeast two hybrid screens which are often confirmed as false positives. For the results of this study these proteins were not seen as interesting candidates so were not validated by other methods. 10 colonies were initially thought to be the KA1 domain of Ppk9. However, further sequencing showed these clones had been inserted in the vector incorrectly to give a nonsense construct. Direct testing showed no interaction between any of the KA domains (data not shown). The transcription factor eIF5A gene *tif51* gave one positive colony, although this is a protein which is highly abundant and is likely to be a false positive (personal communication Colin Gordon). Of

	Ssp2 KA1			Ppk9 KA1			Kin1 KA1			Chk1 KA2		
	-LW	-LWH	-LWHA	-LW	-LWH	-LWHA	-LW	-LWH	-LWHA	-LW	-LWH	-LWHA
Empty	Y	N	N	Y	N	N	Y	N	N	Y	N	N
Nuc2	Y	N	N	Y	N	N	Y	N	N	Y	N	N
Cut4	Y	N	N	Y	N	N	Y	N	N	Y	N	N
Cut9	Y	N	N	Y	N	N	Y	N	N	Y	N	N
Cut20	Y	N	N	Y	N	N	Y	N	N	Y	N	N
Cut23	Y	N	N	Y	N	N	Y	N	N	Y	N	N
APC5	Y	N	N	Y	N	N	Y	N	N	Y	N	N
APC10	Y	N	N	Y	N	N	Y	N	N	Y	N	N
APC11	Y	N	N	Y	N	N	Y	N	N	Y	N	N
APC13	Y	N	N	Y	N	N	Y	N	N	Y	N	N
APC14	Y	N	N	Y	N	N	Y	N	N	Y	N	N
Hcn1	Y	N	N	Y	N	N	Y	N	N	Y	N	N
Rev7	Y	N	N	Y	N	N	Y	N	N	Y	N	N
Mad2	Y	N	N	Y	N	N	Y	N	N	Y	N	N
Mad3	Y	N	N	Y	N	N	Y	N	N	Y	N	N
Bub3	Y	N	N	Y	N	N	Y	N	N	Y	N	N

Table 2: Results of Direct Yeast Two Hybrid Between KA Domains and APC/C Subunits.
KA domains from all four kinases were cloned into pGAD424 and pGBT9 yeast two hybrid vectors along with subunits of APC/C and the MCC. Interactions were tested by co-transforming the vectors into J69-4A. To test for successful transformation the cells were spotted onto SC-LW as well as SC-LWH to test weak interaction and SC-LWHA to identify strong interaction. SNF1 and SNF4 were used as positive controls and each construct was tested for self activation with the corresponding empty vector. Y represents grown whilst N indicates no growth on the selective media indicated.

Bait	Hit	Gene Name	Function	No Hits
Ssp2	SPCC1919.03c	-	AMP-activated protein kinase beta subunit	1
Ppk9	SPAC1002.13c	psu1	Cell wall protein Psu1, beta-glucosidase	1
Chk1 KA2	SPAPJ698.02c	rps002	40S ribosomal protein S0B	32
Chk1 KA2	Non sense construct	-	-	10
Chk1 KA2	SPBC685.07c	rpl27-1	60S ribosomal protein L27	1
Chk1 KA2	SPAC22H12.04c	rps1-2	40S ribosomal protein S3a	1
Chk1 KA2	SPCC1393.03	rps15-1	40S ribosomal protein S15	1
Chk1 KA2	SPAC521.05	rps8-2	40S ribosomal protein S8	1
Chk1 KA2	SPAC806.03c	rps26-1	40S ribosomal protein S26	1
Chk1 KA2	SPBC18E5.06	rps21	40S ribosomal protein S21	1
Chk1 KA2	SPAC23C11.02c	rps23	40S ribosomal protein S23	1
Chk1 KA2	SPBC17G9.07	rps24-2	40S ribosomal protein S24	1
Chk1 KA2	SPCC24B10.09	rps17	40S ribosomal protein S17	1
Chk1 KA2	SPBC18E5.04	rpl10	60S ribosomal protein L10	1
Chk1 KA2	SPCP20C8	chromosome 3	-	1
Chk1 KA2	SPBC83.17	mbf1	Transcriptional coactivator, multiprotein bridging factor	1
Chk1 KA2	SPAC922.04	sequence orphan	sequence orphan	1
Chk1 KA2	SPBC1815.01	eno1	Enolase	1
Chk1 KA2	SPAC26H5.10c	tif51	Translation elongation factor eIF5A	1
Chk1 KA2	SPAC7D4.07c	trx1	Cytosolic thioredoxin	1
Chk1 KA2	SPAC26F1.06	gpm1	Monomeric 2,3-bisphosphoglycerate (BPG)-dependent phospho-glycerate mutase (PGAM)	1
Chk1 KA2	SPBC25H2.05	egd2	Nascent polypeptide-associated complex alpha subunit	1
Chk1 KA2	SPAC1782.07	qcr8	Ubiquinol-cytochrome-c reductase complex subunit 7	1
Chk1 KA2	SPBC83.01	ucp8	UBA/EH/EF hand domain protein	1
Chk1 KA2	SPBC119.02	ubc4	Ubiquitin conjugating enzyme	1
Chk1 KA2	SPAC11G7.04	ubi1	Ribosomal-ubiquitin fusion protein	1
Chk1 KA2	SPAC821.08c	slp1	Sleepy homolog Slp1	2

Table 3: Results of Yeast Two Hybrid Screen Between KA Domains.

pGBT9 containing KA domains were transformed into J69-4A cells and selected for on SC-W. Cells were then transformed with Beach cDNA library and selected for strong interaction on SC-LWHA. After re testing on fresh SC-LWHA DNA was extracted and the insert amplified by PCR in order to be sequenced and identified.

the positive colonies analysed, the most likely to be true positives include another gene linked to transcription *mbf1*, a transcription factor co activator. A pair of interesting hits were *gpm1* which encodes monomeric 2,3-bisphosphoglycerate (BPG)-dependent phosphoglycerate mutase (PGAM) and *eno1* which encodes enolase, two enzymes that act consecutively in the glycolysis pathway (Sternberg et al. 1981; Moreno-Sánchez et al. 2008). *trx1* thioredoxin-1 also produced one hit and is involved in redox reactions that protect the cell from stress (Holmgren & Lu 2010). Whilst the gene *qcr8* is a subunit of ubiquinol-cytochrome-c reductase which is found in mitochondria and is involved in electron transport (Trumpower & Edwards 1979).

Several of the positive interactors appear to have links to the Ubiquitin Proteasome System (UPS). Although the alpha subunit of the Nascent polypeptide-Associated Complex (NAC) Egd2 has not been well studied in *S. pombe*, a search of the database BioGRID revealed that the *S. cerevisiae* homologue (with the same name) has been shown to interact with ubiquitin, several subunits of the proteasome and the E2 conjugating enzyme Ubc4 (BioGRID 2010a). Interestingly *S. pombe ubc4+* was also identified in our screen. This E2 has many genetic links to APC/C and the proteasome in *S. cerevisiae* (BioGRID 2010a) which may suggest that Chk1 has a role in linking the NAC to the UPS. Two other ubiquitin related proteins include UBA/EH/EF hand domain protein encoded by *ucp8* which also contains a UBA domain (Hartmann-Petersen, C. A. M. Semple, Ponting, et al. 2003b) as well as ribosomal-ubiquitin fusion protein gene *ubi1*.

The most interesting interaction was the APC/C co-activator *slp1* which gave the 3rd greatest number of hits, which may explain how the KA domains interact with APC/C. Since Slp1 is not a core subunit; it may also account for the specific binding of APC/C containing unphosphorylated Cut9. For this reason Slp1 was the sole candidate from this screen used for further study. According to the BioGRID (2010b) database this is the first time a kinase has been shown to interact with Slp1 via yeast two hybrid. *slp1* has already been linked by phenotypic suppression to *rad3*, the kinase responsible for activating Chk1 at the beginning of the DNA damage checkpoint (Sugimoto et al. 2004).

All of these interactions appear to be novel. This is most likely due to the fact that to date most yeast two hybrid screens have been performed using full length protein, not just the KA2 domain, which would explain why none of these genes have been identified before.

No colonies were seen for Kin1 KA1 and only one colony appeared for both Ssp2 and Ppk9. The Ssp2 KA1 interactor was identified as the β-subunit of *S. pombe* AMPK which was predicted to interact with the KA1 domain of Ssp2 from the crystal structure of AMPK (Townley & Shapiro 2007). Ppk9 KA1 was shown to interact with β-glucosidase cell wall protein Psu1, which appears to be a novel interaction which needs further validation.

3.4 The KA Domains Bind Directly the APC/C Co-Activator Slp1

Expression of GST-Slp1 proved difficult in bacteria and resulted in multiple bands being products when analysed by SDS PAGE, a common problem seen with Slp1 homologues from other organisms (Sironi et al. 2001). It has been speculated that this may be due to Slp1 requiring chaperones to allow the protein to fold correctly. However, a method devised that induces protein at late log phase and also involves cold shock (2.6.2) was tested since it had been demonstrated to produce proteins which are otherwise difficult to purify from bacteria (Galloway et al. 2003). This method showed good induction of a single band GST-Slp1 fusion protein (Figure 13 **a**, arrow). The GST coupled beads were consequently used to test for interaction with whole cell lysate from *S. pombe* cells expressing HA tagged Ssp2, Ppk9 and Chk1. After separation on 10% SDS PAGE gels and transferred onto membranes, samples were blotted with αHA antibody to detect the presence of the KA containing kinases. Of the kinases tested only Chk1-HA was able to be recognised and bound by recombinant GST-Slp1 (Figure 13 **b**)

Possible explanations as to why Ssp2 and Ppk9 HA proteins were not bound by GST-Slp1 were i) the HA tag hinders the interaction, ii) the recombinant GST protein has not

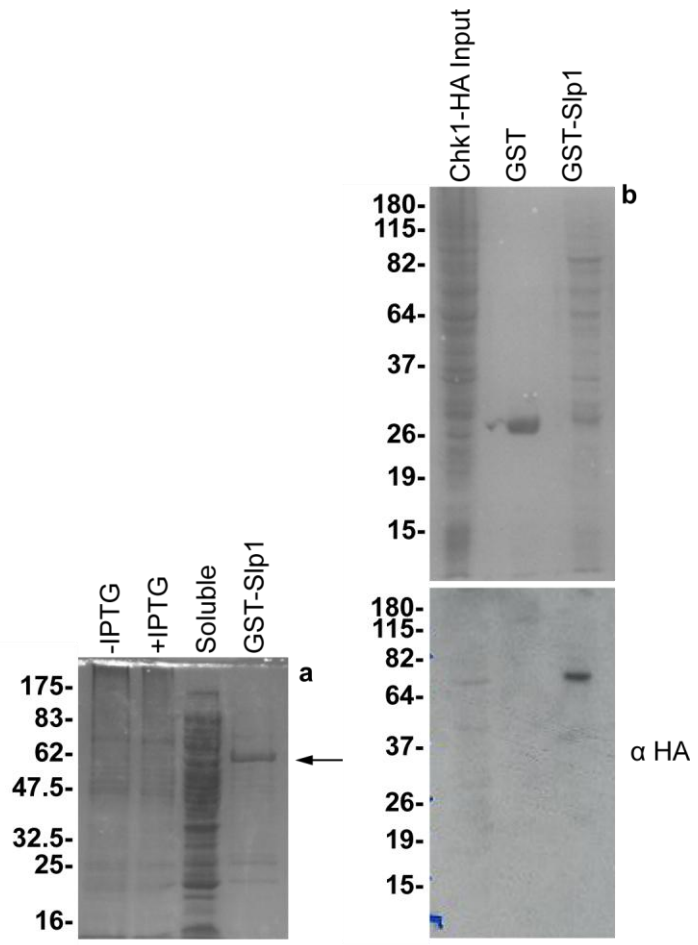


Figure 13: Binding if Chk1-HA by GST-Slp1.

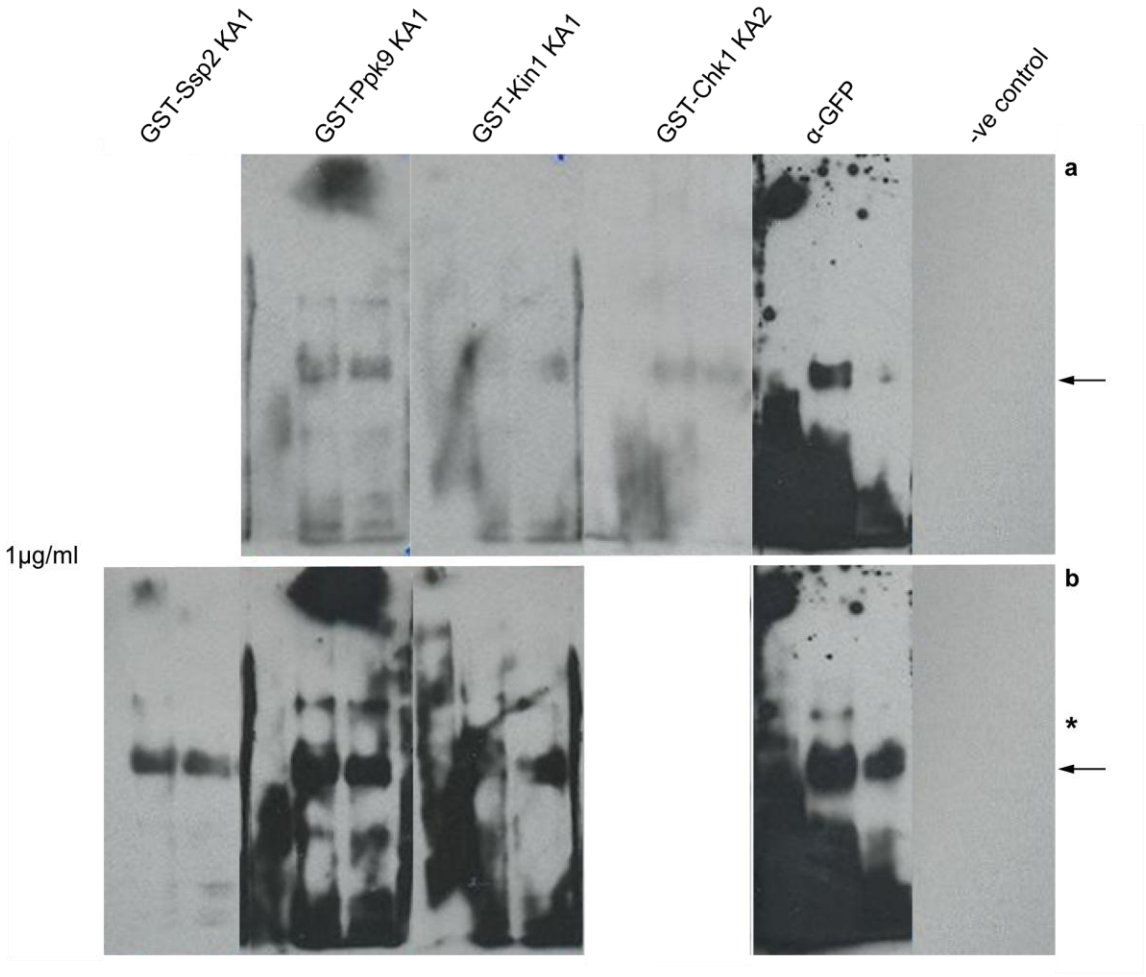
a) Coomassie stained 10% SDS PAGE gel of expressed GST-Slp1 (arrow) using log phase method (see section 2.6.2). **b**) GST-Slp1 expressed in bacteria and purified onto sepherose beads was incubated with lysate from a *S. pombe* strain containing a HA tagged form of Chk1. Excess protein was removed by washing and the proteins separated on 10% SDS PAGE. The top panel shows Coomassie staining of the input GST proteins whilst the bottom panel shows Western blot analysis using anti HA. GST-Slp1 interacted and bound to Chk1-HA whilst GST alone could not.

folded properly and iii) Slp1 is unable to interact with these proteins when it is bound to APC/C. To test this further, a native far Western was performed by making native whole cell lysate from *GFP-slp1* strain. 100µg and 50µg of total extract was loaded and separated using native PAGE. The proteins were then transferred onto a membrane and blocked over night in either dilution buffer or 5% BSA. Purified GST fusion KA domain proteins were then added at a concentration of 1µg per ml to 4µg per ml. No GST protein was added to the negative control and 1:1000 αGFP was used to visualise the GFP-Slp1 fusion protein. A αGST HRP was used as a secondary antibody 1:100,000 for test substrates and the negative control, and α mouse HRP was used for the GFP control.

The results in Figure 14 show that the native far Western negative controls were blank, proving that the αGST HRP antibody could not recognise any protein in the lysate. The αGFP positive control showed a single band at lower exposure (Figure 14 **a**), with a higher molecular weight band of lower concentration appearing at higher exposure (Figure 14 **b**), indicating the position of GFP-Slp1. All four GST-KA domains strongly bound to a single band of the same size recognised by the αGFP control, some showed weaker binding to a band consistent with that also seen in the αGFP control for 1µg per ml. Therefore, all four KA domains are able to recognise and bind to Slp1. Higher concentrations of KA proteins were tested for Ppk9, Kin1 and Chk1 (Figure 14 **c**, **d** & **e**); again all bound to a band of the same size recognised by the αGFP-Slp1 control. Since whole cell lysate from *GFP-slp1* strain was used, any other proteins that may bind the KA domains should also be present. The far Westerns probed with the purified KA domains only produced one band; this suggests that Slp1 is the principal target for the KA containing kinases.

3.5 Discussion

KA1 domains have been described in the MARK family of kinases which include *S. pombe* Kin1 (Drewes & Nurse 2003) but as yet, no function has been assigned to this well defined domain. As the crystal structure of more kinases are being established, the


GST-Ssp2 KA1
GST-Ppk9 KA1
GST-Kin1 KA1
GST-Chk1 KA2
α-GFP
-ve control
a
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GST-Kin1 KA1
α-GFP
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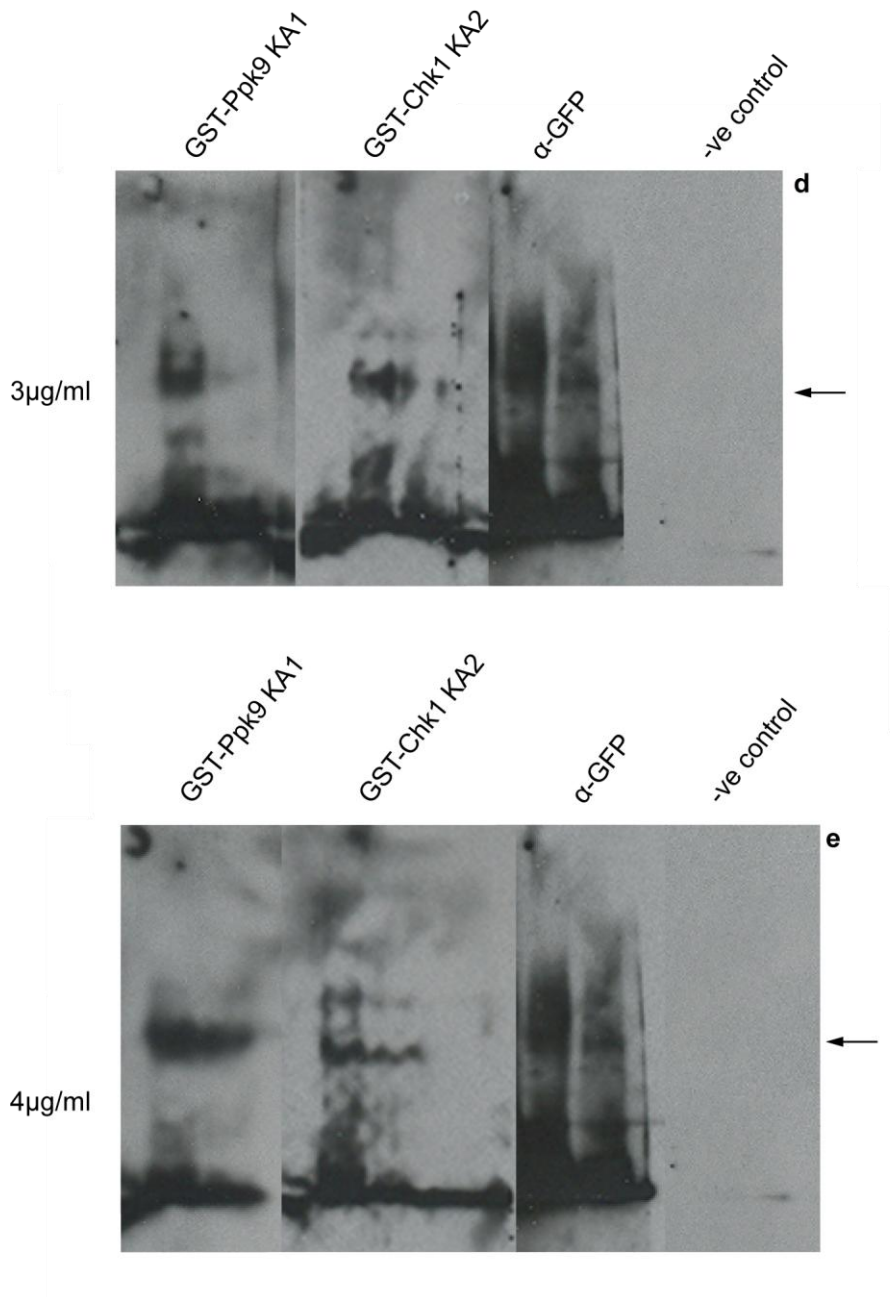


Figure 14: Far Western of GST-KA Protein with GFP-Slp1.

Whole cell lysate from cells expressing GFP-Slp1 were made using non denaturing conditions and run on a NuPAGE® Novec® Tris Acetate 3-8% gels under native conditions and transferred onto a membrane. Far Westerns were then performed with purified GST-KA domain proteins at various concentrations or with anti GFP antibody. Membranes were then probed with HRP GST or secondary antibodies. For a negative control, no protein was added to determine if the anti GST antibody recognised any unspecific proteins in the lysate. **a**) When 1μg/ml of GST-KA protein was used to probe, a shorter exposure showed

that the KA domains of Ppk9, Kin1 and Chk1 all bound a single band of the same size, which was equivalent to the band produced by the GFP antibody (arrow); however Ssp2 KA did not produce a band at this level of exposure. **b**) A longer exposure of the same blot produced two bands (asterisks and arrow) for the KA domains of Ssp2, Ppk9 and Kin1. Again these were the same size as the GFP control which also produced two bands. At this level of exposure the KA2 domain of Chk1 was over exposed. **c**, **d** & **e**) Higher concentration of protein from 2-4µg/ml were used to test Ppk9KA1, Kin1KA1 and Chk1 KA2, which all recognised and bound a band which was also recognised by the GFP antibody. In all experiments the negative control of no primary protein followed by the HRP GST secondary antibody was blank, proving that the bands seen were not due to unspecific antibody binding but as a result of the KA proteins recognising a substrate.

number of proteins described as having a KA1 domain has increased due to the structural motif rather than the sequence of the domain. As a result, the definition of the KA1 domain has expanded. Up to this point the function of the KA domain has remained elusive; however, it has been speculated that this domain could be involved in localisation or have a role in regulation of the N-terminal kinase domain (Guo & Kemphues 1996; Beullens et al. 2005). The data presented provide *in vitro* evidence that one of the functions of the KA domains is to allow a subset of kinases to interact with APC/C.

Cut9 is only phosphorylated when it is part of the 20S APC/C complex and not in a free form. Synchronised cells of the *cut9-HA* strain showed that the upper phosphorylated band was only present in metaphase cells - a stage in the cell cycle when APC/C is most active. However genetic studies showed that the double mutant of *cut9*-665 with the fission yeast PP1 phosphatase mutant *dis2-11* was lethal. Alternatively, the double mutant *cut9*-665 *pka1*Δ that lacked the catalytic subunit of the PKA kinases could rescue the temperature sensitive phenotype of the *cut9-665* mutant (Yamada et al. 1997). This suggests that PP1 positively regulates APC/C activity whilst PKA inhibits activity, although direct interactions were not proven. Regardless, this data showed that the phosphorylation state of Cut9 is important in regulating APC/C activity. Since the KA domains specifically bind APC/C with unphosphorylated Cut9 there must be something specific, such as a conformational change or co-activator, that the domains can recognise.

The direct yeast two hybrid results rule out an interaction with core APC/C subunits or the MCC. However, a library screen revealed that the KA2 domain of Chk1 could interact with the co-activator Slp1, and far Westerns confirmed that the KA domains all bound to a protein the same size as native GFP-Slp1. Although other proteins were identified in the library screen, only binding to the GFP-Slp1 band was observed in the native far Westerns which would indicate that Slp1 is the main or preferred target for KA domain interaction. This was a surprising result indicating that the phosphorylation state of Cut9 has some bearing on the ability of Slp1 to bind to APC/C. In budding yeast,

mutations in the Cut9 homolog CDC16, that converted the phosphorylation sites to alanine, produced an APC/C complex with severely reduced ability to bind the Slp1 homolog CDC20. Similar mutations to the Nuc2 subunit CDC27 reduced binding to a lesser degree (Rudner & Murray 2000). Taking these findings into account, it may be that in *S. pombe*, the KA containing kinases bind to APC/C (which contains unphosphorylated Cut9) through Slp1 which is bound to Nuc2. Further investigation may identify the link between the Cut9 phosphorylations effect on APC/C activity and the interaction with Slp1 in fission yeast.

This is the first observed direct interaction of a KA domain with another protein and is the first confirmed role of the KA domain. However, the crystal structure of Ssp2 in the *pombe* AMPK complex suggested an additional function of the KA1 domain is to interact with the β subunit (Townley & Shapiro 2007). Interestingly the yeast two hybrid results are consistent with this function. This would lead to the conclusion that the KA domains are involved in other interactions which as yet remain unidentified. The KA domain of Kin1 did not yield any positive colonies, even through the best defined KA domain is that of Kin1. Kin1 KA1 domain also appears to have a stronger interaction with APC/C from the Cut9-HA Western blots.

Chk1 KA2 domain is slightly different to the KA domains of Ssp2, Ppk9 and Kin1 and may account for the fact that it yielded many more interactions and indeed the only KA domain that gave a positive interaction with Slp1 in the yeast two hybrid screen. Chk1-HA protein interacted with GST-Slp1 whereas Ssp2 HA and Ppk9 HA proteins could not. Perhaps the Chk1 KA2 may bind to a different site of Slp1 or the confirmation of Slp1 in the context of APC/C is important for KA1 domain binding. This theory is supported by the native far Western results which show all KA domains could bind GFP Slp1 in its native form. However, further analysis of the binding site in Slp1, which the KA domains interact with, is required to deduce if the two different types of KA domains recognise the same motif.

In all analyses, there are always the limitations of the techniques used and the yeast two hybrid is no exception, and may account for Kin1 KA1 domain being unable to interact and Ssp2 and Ppk9 KA1 domains yielding a low return. The cDNA library used may have an under representation of those proteins expressed at low levels, or very large proteins whose RNA's reverse transcribes inefficiently and produce incomplete proteins. To propagate a cDNA library constructs may be lost and high stringency of selection may not identify transient interactions. It should be noted that the two hybrid screen is carried out in *S. cerevisiae*, and therefore binding partners or post-translational modifications may not occur and interaction partners cannot be observed and identified.

It is known that the Chk1 C-terminus is well conserved and is essential for controlling kinase function as well as its role in the DNA damage checkpoint (Kosoy & O'Connell 2008; Palermo et al. 2008). Our proposed KA2 domain (residues 323-496) contains the two areas of high conservation (residues 394-400 and 468-477) identified by Kosoy & O'Connell (2008). They showed that point mutations and deletions in these regions can lead to both increases and decreases in kinase activity *in vivo*. They did this by testing the ability of the mutants to rescue the *chk1*Δ phenotype when exposed to MMS. It is possible that the KA2 domain is needed to bind regulators or substrates and that these mutations strengthen or weaken the interactions accordingly. Another controversial theory is that the C-terminus acts as an auto-inhibitory domain. A region of fission yeast Chk1 identified by Palermo et al. (2008) appears to encode a pseudo substrate domain (residues 464-469) which may bind the kinase domain and regulate its activity. However, this theory is not supported by yeast two hybrid data which shows no interaction between the isolated kinase domain and the C-terminus and cannot explain why some of the mutations cause loss of function.

Several of the positive yeast two hybrid proteins have a link to UPS, such as Slp1, Ucp8, Ubc4 and Ubi1. A library screen with full length Chk1 has also identified proteins with links to ubiquitin such as a potential E3 ligase containing a RING finger domain and another protein containing an EF-hand domain (Kosoy et al. 2007). A number of different observations indicate that the DNA damage checkpoint and the control of

cohesin cleavage appear to be linked; the double mutant of a compromised *cut2*^{EA2} and *chk1*Δ has an increased sensitivity to UV damage than either of the single mutants. In addition, cells carrying a form of Rad21 (a subunit of cohesion) which cannot be cleaved were slower to repair thymine dimers after UV radiation compared to WT (Nagao et al. 2004). If the binding of Chk1 to APC/C, which is ultimately responsible for the destruction of Rad21 though Cut2, allows Chk1 to regulate its activity, it could influence when Cut2 gets ubiquitinated and could delay or bring forward the cohesin cleavage which separated the sister chromatids allowing the DNA to be repaired.

Chapter 4
Phosphorylation of Mad2

4.1 Introduction

Unlike some E3 ligases such as SCF which are periodically activated, APC/C can always recognise its substrates, however, its activity is tightly regulated by phosphorylation and inhibition by the Mitotic Checkpoint Complex (MCC) (Vodermaier 2004). Kraft et al. (2003) demonstrated that in human APC/C, there are over 43 sites of phosphorylation and that 34 are specific to mitosis. It has also been shown that adding phosphatases to active APC/C preparations results in a reduction of ligase activity (Kramer et al. 2000).

Multiple subunits of APC/C are phosphorylated at several sites by cyclin dependent kinases (Cdks). Cdk phosphorylation of APC/C between G2 to M phase is required to bind Cdc20, the homologue of co-activator Slp1, and causes activation of APC/C^{Cdc20} at prophase through to anaphase. The reduction of Cdk activity at mid anaphase causes the formation of APC/C bound to the second co-activator Cdh1 (Ste9 in *S. pombe*). Cdh1 can bind APC/C regardless of the enzymes phosphorylation state. However, when Cdh1 is phosphorylated by Cdk1-CyclinB this causes dissociation from APC/C at late G1 to S phase and corresponds to a loss of ligase activity (Vodermaier 2004; Skaar & Pagano 2009).

Phosphorylation has been shown to have both a positive and negative effect on APC/C activity. In fission yeast PKA phosphorylates the Cut4 and Nuc2 subunits of APC/C causing inaction of the complex. Phosphorylation of Cut9 has also been shown to be important for regulating APC/C activity at metaphase (Yamada et al. 1997; Acquaviva & Pines 2006). Cdk1-CyclinB (Cdc2-Cdc13 in *S. pombe*) and Plk (Plo1 in *S. pombe*) have been shown to phosphorylate APC/C subunits including Cdc27 (*S. pombe* Nuc2), Cdc23 (*S. pombe* Cut23), Cdc16 (*S. pombe* Cut9) and *S. pombe* Hnc1 (Rudner & Murray 2000; C. Kraft et al. 2003; Yoon et al. 2006). Hnc1 is an essential subunit needed to assemble the APC/C complex and is phosphorylated at serine 48 by Cdc2-Cdc13. To

show this phosphorylation is critical for function, mutations were made to change serine 48 to alanine which resulted in a delay in the final stage of cell division and causes an increase in cells which have not separated properly (Yoon et al. 2006).

APC/C is also regulated by the MCC, usually made up of Mad2, Mad3 and Bub3 (Sczaniecka et al. 2008). During metaphase to anaphase conversion the kinetochores of the chromosomes undergo bipolar attachment to microtubules in order to be separated into daughter cells. Unbound kinetochores are recognised by the spindle checkpoint. In this checkpoint the Mitotic Arrest Deficiency 1 (Mad1) protein binds to kinetochores and recruits the Mad2 protein. Mad2 exists in two different states, the open O-Mad2 or closed C-Mad2. O-Mad2 is in a kinetically higher energy confirmation and cannot bind Mad1 or the Slp1 homologue Cdc20. O-Mad2 can bind to C-Mad2 in a dimer complex with Mad1 at the kinetochore and is converted from O-Mad2 to an intermediate I-Mad2. This intermediate protein converts to C-Mad2 which dissociates from C-Mad2- Mad1 dimer and can then bind Mad1 or Cdc20 attached to APC/C (Yang et al. 2008). The binding of C-Mad2 to Cdc20 initiates the formation of the MCC which inhibits APC/C activity stopping the progression through to anaphase until all the kinetochores are bound to microtubules.

To reverse the inhibition of APC/C the cell utilises two different mechanisms. The protein Cmt2/p31^{COMET} binds to C-Mad2 in the Mad1 dimer blocking its ability to bind and convert O-Mad2 and therefore Mad2 cannot be loaded onto APC/C. In addition p31^{COMET} has been shown to bind to C-Mad2 attached to APC/C and causes auto-ubiquitination and degradation of Cdc20 bound to C-Mad2 which leads to activation of the E3 (Reddy et al. 2007; X. Luo & Yu 2008). Mad2 is also phosphorylated at several serine residues at its C-terminus which renders it unable to bind Cdc20 and causing it to dissociate from APC/C. It has also been demonstrated that mutations that mimic phosphorylation of Mad2 at several serine residues causes the protein to adopt the open confirmation. In addition phosphorylation of human Cdc20 at S195 prevents conversion to C-Mad2 (Wassmann et al. 2003; Kim et al. 2010). When APC/C is released from inhibition it is then free to act on targets which promote the onset of anaphase.

All four KA containing kinases are involved in processes which are important for cell division. Chk1 is part of the DNA damage pathway which blocks the cells passage from G2 to M phase in response to DNA damage (Walworth et al. 1993). Chk1 has also been implicated with the spindle checkpoint (Zachos et al. 2007). Ssp2 and Ppk9 are part of the 5' AMP-activated Protein Kinase (AMPK) complex involved in regulating metabolism and are activated during times of glucose limitation, conditions which are not favourable for cell division (Hanyu et al. 2009), whilst Kin1 regulates microtubule stability and cell growth (Drewes & Nurse 2003).

Although not much is known about Ssp2 and Ppk9 in fission yeast, their orthologue in *S. cerevisiae*, Snf1, has been well studied due to AMPKs implication in diabetes (Iseli et al. 2008). Snf1 is the α catalytic subunit which along with the γ regulatory Snf4 and either one of the β subunits Sip1, Sip2 or Gal38 forms the heterotrimer enzyme SNF1. Studies have shown that along with an N-terminal kinase domain, Snf1 contains a C-terminal regulatory domain which can bind to and inhibit the kinase domain in the presence of high amounts of glucose. However, when glucose levels are low, this domains ability to bind the kinase domain decreases and instead, it interacts with the γ regulatory subunit Snf4 leading to activation of the SNF1 complex. Interestingly, genetic and biochemical studies have shown that the Snf1 regulatory domain is needed for the formation of the SNF1 complex, although Snf1 on its own does retain some kinase activity which can be increased by removal of the regulatory domain (Hardie et al. 1998; Elbing et al. 2006).

The C-terminus of Chk1 is also very important in regulating its own N-terminal kinase domain. Kosoy & O'Connell (2008) demonstrated that the deletion of the last 11 amino acids of Chk1 C-terminus was enough to cause inactivation of Chk1 kinase activity. Consistently many mutations in the C-terminus have been shown to effect kinase activity. Exactly how this happens is still controversial. One of the most attractive hypotheses is that the C-terminus interacts with the kinase domain and inhibits its activity in a similar manner to Snf1. A pseudosubstrate domain was identified in the C-terminus inside our proposed KA2 domain in which the phosphoacceptor site had been replaced with a conserved aspartic acid. This motif should be recognised by the kinase

domain of Chk1 providing a possible mechanism for auto-inhibition (Palermo et al. 2008). However mutations have been made to the C-terminus that decreased activity which contradicts this model. Also yeast two hybrid assays failed to show interactions between the two domains suggesting that the C-terminal does not provide a mechanism for regulation (Palermo et al. 2008; Kosoy & O'Connell 2008; Tapia-Alveal et al. 2009).

Since all four of these protein kinases are capable of binding APC/C, it was important to investigate if they could phosphorylate, and therefore regulate, APC/C. The results in this chapter show that the kinases can phosphorylate a ~30kDa protein associated with APC/C, and that this protein is Mad2, part of the MCC complex not APC/C. This data also suggests that the KA domains may have an additional role in regulating kinase activity by acting as auto-inhibitors to the N-terminal kinase domains.

4.2 Full Length Proteins Phosphorylate a 30kDa band of Tap Tagged Purified APC/C

In order to investigate if the KA containing kinases were involved in regulating APC/C activity the APC/C complex was purified from an integrated Tandem Affinity Purification (TAP) tag *cut20/lid1* strain. The TAP tag consists of a Calmodulin Binding Peptide (CBP) and Protein A separated by a Tobacco Etch Virus (TEV) protease cleavage site. The APC/C complex was purified as described in Material and Methods 2.6.17. The complex was then separated on SDS PAGE gels and silver stained which gave a similar banding pattern to that previously published (Figure 15 **a**, Appendix B) (Yoon et al. 2002). GST tagged full length Ssp2, Ppk9 and Kin1 where bacterially expressed and purified by coupling to Glutathione Sepharose™ 4B beads before eluting using L-glutathione in order to test for kinase activity.

First the activity of the kinases was tested by looking for autophosphorylation by incubating with radiolabelled ATP at Universitätsmedizin Berlin in collaboration with

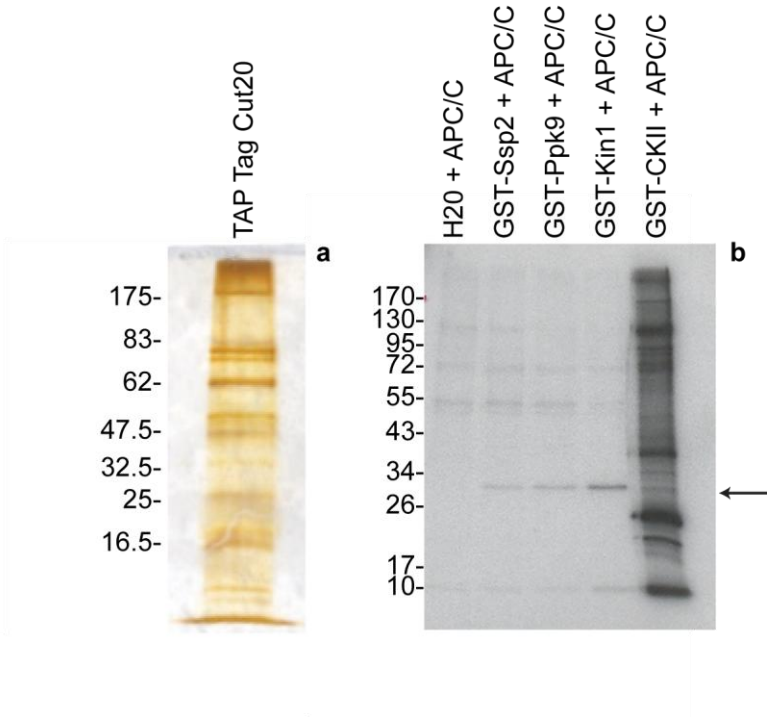


Figure 15: Phosphorylation of Purified Tap Tagged Cut20 APC/C by Full Length Proteins.

a) A strain in which endogenous *cut20+* has been replaced with a TAP tag form was used to purify APC/C and visualised using silver staining. This complex was then used to perform phosphorylation assays with radio labelled ATP and bacterially expressed GST fusion proteins of Ssp2, Ppk9, Kin1 and CKII. **b**) The three KA containing kinases phosphorylated a protein of ~30kDa which was not present in the water control. CKII was used as a positive control for the assay.

Professor Wolfgang Dubiel. Proteins were then separated by SDS PAGE and transferred to a membrane which was exposed to x-ray film. This result showed that none of the kinases appeared to be active (data not shown). However this could be due to the KA domain inhibiting the N-terminal kinase domain. The kinases, along with CKII as a positive control, were incubated with APC/C and radiolabelled ATP then separated on SDS PAGE gel before transferring onto membranes. Overnight exposure showed that CKII phosphorylated several proteins whilst the three kinases only phosphorylated one specific 30kDa protein which was not seen in the water control (Figure 15 **b**). However, the molecular weight of this protein does not correspond to any of APC/Cs core 13 subunits or its co-activators suggesting that these kinases act on a protein associated to APC/C.

4.3 Ssp2 and Ppk9 Phosphorylate Mad2

Previous published work has used the *cut20 TAP* tag strain to purify APC/C in order to identify new subunits and proteins transiently associated with APC/C. NMR analysis showed that along with the co-activator Slp1, Mad2 and Mad3 of the MCC were also purified with the complex. The third MCC subunit Bub3 is not present in *S. pombe* APC/C-MCC complex because fission yeast Mad3 lacks the motif that bind Bub3 present in other organisms. In these preparations of APC/C Mad2 was identified by Western blots and appeared to migrate at a molecular weight of ~30kDa (Sczaniecka et al. 2008). Since the formation of the MCC is regulated by phosphorylation of Mad2, this made Mad2 a candidate for phosphorylation by KA containing kinases.

Full length and ΔKA GST fusion proteins were purified from bacteria and eluted from beads. They were again tested for their ability to autophosphorylate in collaboration with Professor Wolfgang Dubiel's group, and as shown in Figure 16, both Ssp2 and Ppk9 full length proteins could not autophosphorylate, but the ΔKA proteins could. This is consistent with the hypothesis that the KA1 domain may be acting as an auto-inhibitory

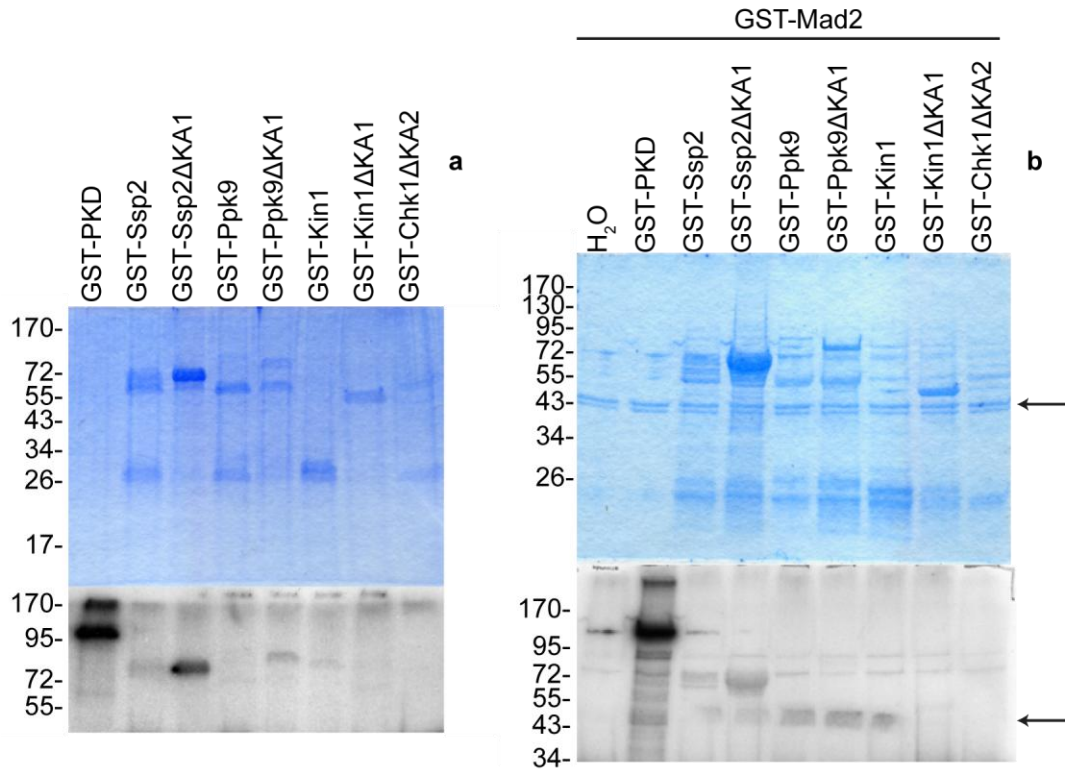


Figure 16: Autophosphorylation of Full Length and ΔKA Proteins and Phosphorylation of Purified GST Mad2.

Full length and ΔKA GST tagged proteins were purified from bacteria extracts onto Glutathione Sepharose™ 4B beads and eluted. They were tested for their ability to autophosphorylate by addition of radiolabelled ATP. **a**) Ssp2 and Ppk9 appear to be able to autophosphorylate in the absence of the KA domains, but not in the presence of the full protein (bottom panel). **b**) The ability of full length and ΔKA proteins to phosphoylate GST-Mad2 was tested by a similar assay. Ssp2, Ppk9 and Kin1 appear able to phopshorylate Mad2 (arrow)

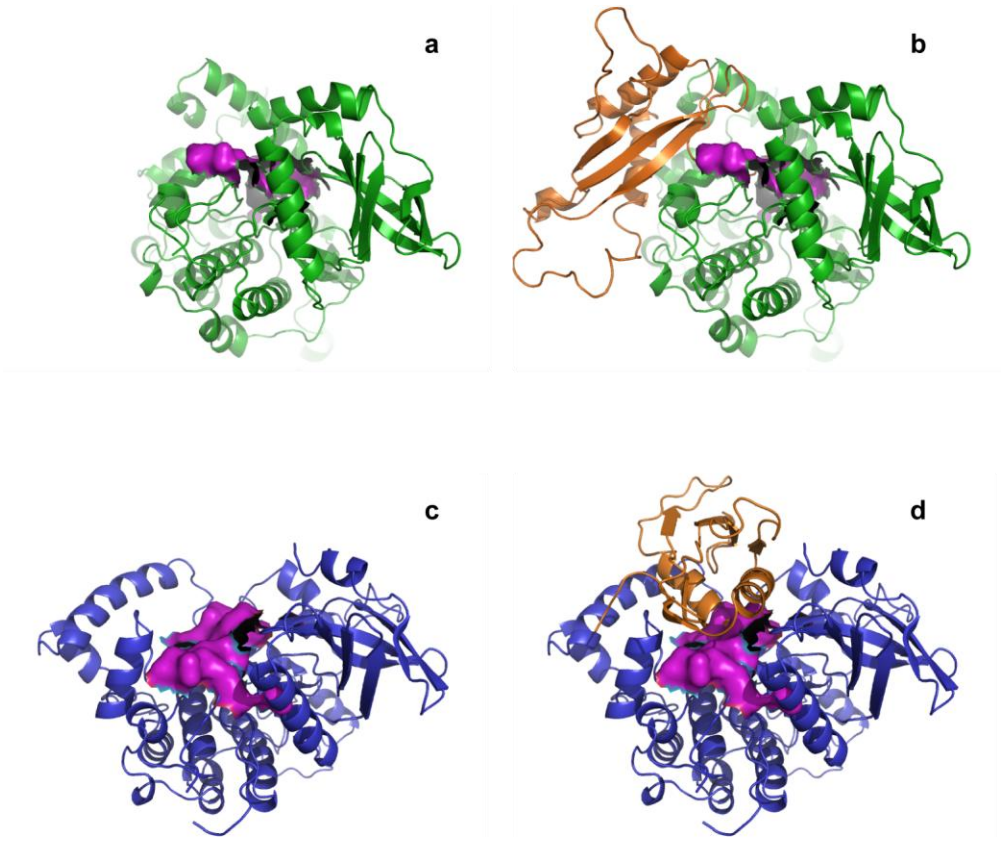


Figure 17: Structure of Ssp2 and Ppk9.

a) The structure of Ssp2 based on the solved crystal structure (Townley & Shapiro 2007) shows the binding site in magenta and everything apart from the KA1 domain in green. **b**) When the KA domain is added, it appears to shut off the access to the kinase binding surface. **c**) Although Ppk9 structure has not been solved, a model based on its sequence and structure of similar proteins show the binding site in magenta and everything except the KA domain in blue. **d**) Again the KA1 domain seems to restrict access to the binding site. This may suggest that the KA1domains act as an auto-inhibitor of the N-terminal kinase. Modelling performed by Konstantinos Paraskevopoulos (MRC HGU)

domain for the N-terminal kinase domain. The structure of the AMPK subunits Ssp2 and Ppk9 seem to support the auto-inhibition hypothesis. Modelling based on the crystal structure of the *S. pombe* Ssp2 as part of the AMPK complex solved by Townley & Shapiro (2007) was performed by Konstantinos Paraskevopoulos (MRC HGU) and shows that the protein without the KA domain has a binding site (Figure 17 **a** magenta) surrounded by α helices except on one side. The KA1 domain occupies this free space and appears to close off access to the ATP binding site (Figure 17 **b**). Although the structure of Ppk9 has yet to be determined, hypothetical modelling was performed based on similar proteins (Figure 17 **c** & **d**). Again it appears that the binding site is blocked by the KA1 domain, lending weight to the auto-inhibition role of KA1 domain.

Kin1 full length and ΔKA proteins of Kin1 and Chk1 did not appear to be active. When tested with GST-Mad2 the kinases Ssp2ΔKA, Ppk9 and Ppk9ΔKA phosphorylate Mad2. This data also showed that Kin1may possibly phosphorylate Mad2; however this protein appeared to have been degraded by the time it was tested.

4.4 Mad2 is Not Needed for KA Domains to Interact with APC/C

Since we have shown that the KA domains can interact with APC/C via the co-activator Slp1, we wanted to determine if the presence of Mad2 was a requirement for this interaction. To do this the *cut9-HA* strain was crossed to the *mad2*Δ mutant and the APC/C interaction assay repeated with KA domains to see if the presence of Mad2 was important for binding. The KA domains were still able to interact with APC/C containing unphosphorylated Cut9 (Figure 18), which would suggest that any phosphorylation of Mad2 by these kinases is transient and does not require binding to Mad2 itself.

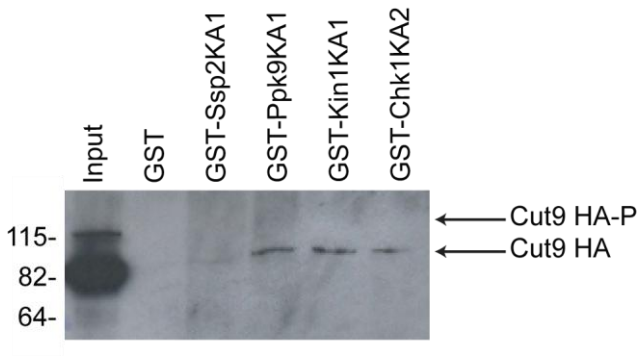


Figure 18: KA Domains Binds to Unmodified Cut9 Form of APC/C in the Absence of Mad2.
Protein extracts from the *cut9-HA mad2*Δ strain produces two bands corresponding to phosphorylated and unphosphorylated Cut9-HA protein. After incubation with GST tagged KA domains excess protein was removed by washing, and samples separated on 10% SDS PAGE gel. Analysis by Western blot using an anti HA antibody revealed that the KA1 domains can still bind the unphsophorylated band exclusively showing that the domains interact with APC/C in a Mad2 independent manner.

4.5 Discussion

The precise role of the KA domain has yet to be defined, although several functions, including localisation and auto-inhibition of the kinase domain, have been proposed (Guo & Kemphues 1996; Beullens et al. 2005). The structure of the AMPK subunits Ssp2, Ppk9 and Chk1 seem to support the auto-inhibition hypothesis. Modelling performed by Konstantinos Paraskevopoulos (MRC HGU) showed that the KA domain of both Ssp2 and Ppk9 appear to block access to the kinase domains binding site (Figure 17). This would be in keeping with the *S. cerevisiae* homologue Snf1, which uses its C-terminus as an auto-inhibitory domain to bind to the N-terminal kinase domain and prevents it from acting on its substrates (Hardie et al. 1998). The C-terminus of the Chk1 protein, in which we have defined a KA2 domain, plays an important role in regulating kinase activity (Kosoy & O'Connell 2008). Although it has yet to be proved conclusively, it is believed that the C-terminus of Chk1 can also bind the N-terminal kinase domain causing inhibition (Palermo et al. 2008).

Initial experiments showed that bacterially expressed GST tagged full length Ssp2, Ppk9 and Kin1 could not autophosphorylate; however, these kinases were then proved to be active when incubated with APC/C (Figure 15). As the KA domains bind to Slp1 this would suggest that the KA domains may be acting as auto-inhibitory domains. Further experiments showed that full length Ssp2 and Ppk9 proteins lacking the KA1 domain could autophosphorylate, but the full length proteins could not (Figure 16). This data would support the notion that a secondary role for the KA domains is to regulate kinase activity. The same results were not seen for Kin1 or Chk1. However, full length Chk1 protein was not available for testing and the preparation of Kin1 full length appears to have degraded every time it was tested, and the two ΔKA proteins did not appear to be active. Further work is needed to confirm that all of the KA domains behave in the same way, preferably using protein expressed in *S. pombe*. These proteins are more likely to be active since they will contain post translational modifications and binding partners which are not present in bacterially expressed protein, and will give a more accurate reflection of the proteins behaviour in the cell.

Chapter 3 presented evidence that the KA domain binds to the APC/C co-activator Slp1, potentially giving kinases access to the E3 ligase APC/C and allowing them to influence the cells transition from metaphase to anaphase. It was therefore important to determine if these KA containing kinases could phosphorylate APC/C. Full length Ssp2, Ppk9 and Kin1 all phosphorylated an ~30kDa protein present in TAP tagged purified APC/C. Previous reports showed that the only protein present in these preparations of this molecular weight was Mad2. The ability of the full length and ΔKA kinases to phosphorylate GST-Mad2 was tested and proved that the active kinases, Ssp2 and Ppk9 could phosphorylate Mad2. It is known that the phosphorylation state of Mad2 controls its ability to form the C-Mad2 conformation and, therefore, controls Mad2 binding to Mad1 and Slp1/Cdc20. However, to date the kinases responsible for these modifications have yet to be identified. This makes the observed phosphorylation by some of the KA containing kinases novel. Therefore, if these kinases phosphorylate Mad2 attached to APC/C this could cause dissociation of the MCC from APC/C. The data also shows that binding of the KA domain to APC/C is not dependent on the presence of Mad2 (Figure 18).

From this evidence we propose that the KA domain can bind to the N-terminal kinase domain of the protein, inhibiting phosphorylation of their substrates. After some cellular event the KA domains can recognise APC/C containing the unphosphorylated Cut9 subunit via the co-activator Slp1. This then leaves the kinase domain free to act on Mad2, which also binds Slp1. Phosphorylated Mad2 then dissociates from Slp1 and cannot bind Mad1 or Slp1 again until it is dephosphorylated. Thus the kinases can control the binding of Mad2 to APC/C and the formation of APC/C^{MCC} and, therefore, can regulate APC/C activity (Figure 19). This is consistent with the involvement of the kinases in pathways needed for cell division. Ssp2 and Ppk9 monitor glucose levels and act on their substrates in times of low glucose, which is an unfavourable condition for cell division. Similar situations exist for Chk1, which is activated after DNA damage allowing cells to repair or halt cell division, and Kin1 regulates microtubule stability, effects cell morphology and cell division. It is plausible to believe that at times when

these kinases are not needed for their primary roles they could be available to bind to and regulate activation of APC/C. On their own these phosphorylation events may not have a drastic effect but each of these kinases may be able to adjust the regulation slightly to delay the segregation of chromosomes, if there is a problem that needs to be resolved.

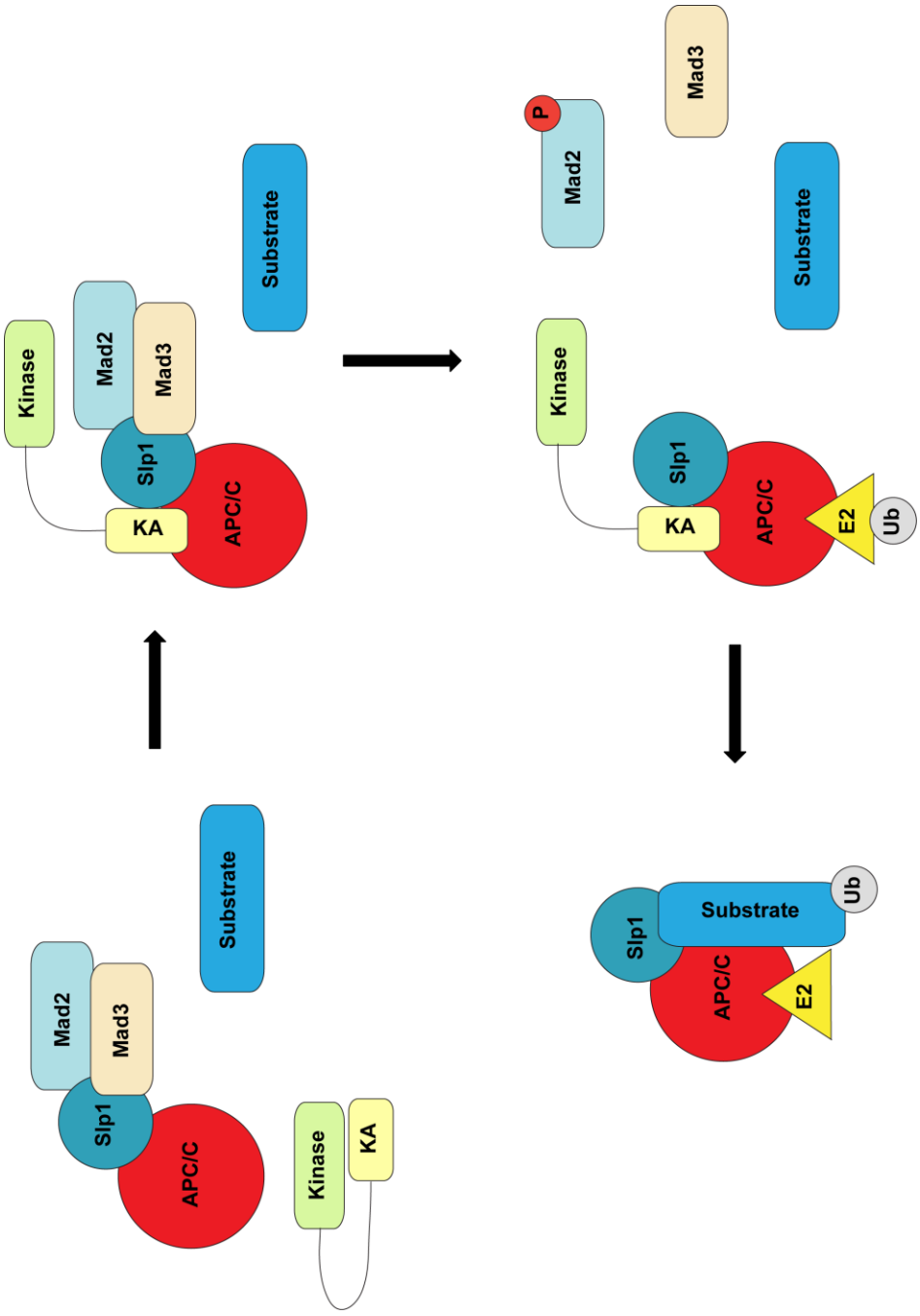


Figure 19: Model of APC/C Regulation by KA Domain Containing Kinases.

A diagram showing the proposed mechanism of the KA domains functions. The KA domains bind to and inhibit the N-terminal kinase domains until some cellular event causes them to recognise and interact with Slp1 attached to APC/C. This then frees the kinase domain to phosphorylate Mad2, causing dissociation of the MCC complex from APC/C. The active complex is therefore released to act on its substrates.

Chapter 5
Characterisation of Kinase Mutants

5.1 Introduction

Post-translational modifications play an important role in altering the function and fate of proteins. Of all the post translational modifications known, phosphorylation of proteins has been the most extensively studied. The ability of kinases to recognise substrates and utilise ATP to transfer phosphate to a serine, threonine or tyrosine residue has been implicated in controlling protein activity in pathways ranging from immunity to behaviour (Tarrant & Cole 2009). To do this, proteins use a catalytic kinase domain which has a well conserved structure which can be used as a marker to identify kinases. The N-terminus of the kinase domain consists of a glycine rich subdomain made of β-sheets and is joined by a linker region to a larger C-terminal subdomain made of α-helices. The pocket created between these two subdomains binds ATP and can be rotated to form either an open or closed confirmation depending on the kinases activational state. This domain also contains conserved residues involved in binding ATP as well as the transfer of phosphate from ATP to the target residue (Scheeff & Bourne 2005).

Systematic study of the genome revealed that *S. pombe* encodes 106 kinases, 47 of which are conserved in humans. Genetic studies showed that 17 of these kinases are essential in fission yeast (Bimbó et al. 2005). Of these 106 kinases, three contain a Kinase Associated 1 (KA1) domains and one possesses a related KA2 domain (personal communication Kay Hofmann).

In order to link the results seen by the *in vitro* biochemistry experiments, performed in the previous chapters, to *in vivo* studies, mutants of all four KA containing kinases were obtained. All four of these null mutants are viable, and therefore are not essential genes. These strains were then subjected to genetic analysis by crossing to different null and *ts* strains. Their response to exposure to different drugs and stresses was also observed. The results, presented in this chapter, did not reveal any phenotype that was exclusively

common to all four kinases which could be exploited as a way to assay the function of the kinases KA domains.

5.2 Phenotype and KA Kinase Mutants

Null mutants of all four kinases have either been published (*chk1*Δ, *ssp2*Δ and *kin1*Δ) (Walworth et al. 1993; Matsusakal et al. 1995; La Carbona et al. 2004) or are commercially available from Bioneer (*ssp2*Δ no:BG-5025 & *ppk9*Δ no:BG-1105). The mutants, *ssp2*Δ, *ppk9*Δ and *kin1*Δ all carry the *G418*R selectable marker. To perform the genetic studies, multiple mutant strains needed to be created, and each mutant type represented with a traceable marker. To this end, *ssp2*Δ and *ppk9*Δ *G418*R markers were changed to *nat*R and *hyg*R respectively via homologous recombination (See Material and Methods 2.3.14) (Sato et al. 2005; Hentges et al. 2005). Figure 20 shows the mutants at 25^{o}C and 36^{o}C. The mutants were then crossed to one another to create every possible variation of double, triple and finally a quadruple mutant, all of which were viable (data not shown, Figure 20). The single mutants were used to express proteins, crossed to other strains or tested for sensitivity against drugs which will be described in the following sections.

5.2.1 *kin1*Δ

*kin1*Δ has the most severe growth phenotype of the four KA containing kinases. In addition to a cell wall defect which causes the cells to stick together, *kin1*Δ cells display a septation defect which leads to the septa forming off centre during cell division. As a result two populations of cells arise; one is small and spherical whilst the other resembles WT cells with one end broader than the other, which has led to these cells being described as “ice-cream cone” shaped (Figure 20) (La Carbona et al. 2004). These cells are slow growing at 25^{o}C with a doubling time of 5 hrs compared to 4 hrs for WT (Figure 22) and also display a *ts* phenotype at 36^{o}C, however, this phenotype appears to be “leaky” and cells can grow for some time before they die (Figure 20 & Figure 21) (Bimbó et al. 2005). Overexpression of YFP tagged Kin1 in WT cells from the Riken

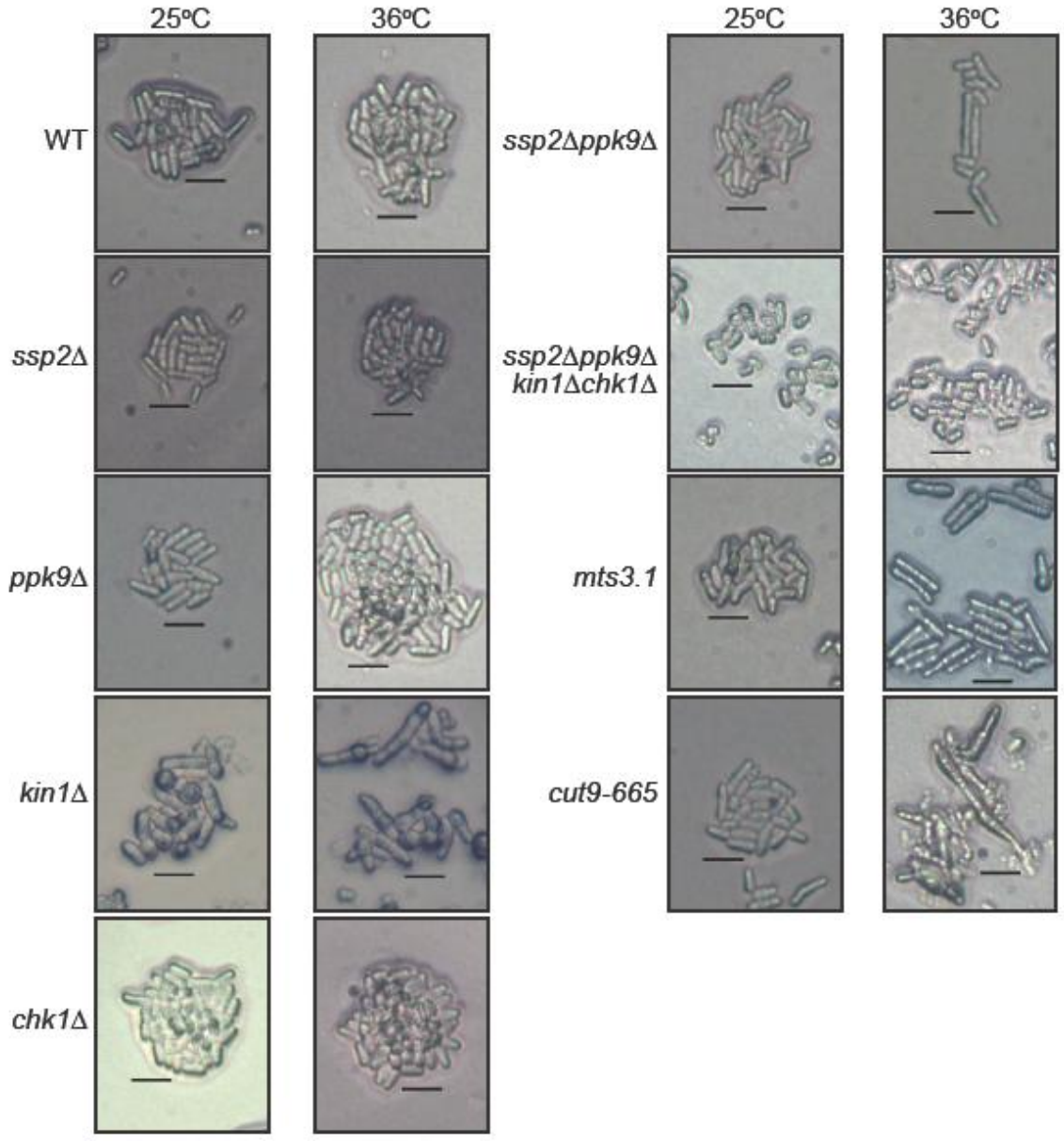


Figure 20: Null and *ts* Mutant Use at 25°C and 36°C.

Colonies of WT and single null mutants of KA containing kinases used in this study along with double and quadruple mutants. *ts* mutants of proteasome *mts3.1* and APC/C *cut9-665* subunits also shown at 25°C and 36°C. Scale bar equals 20μm.

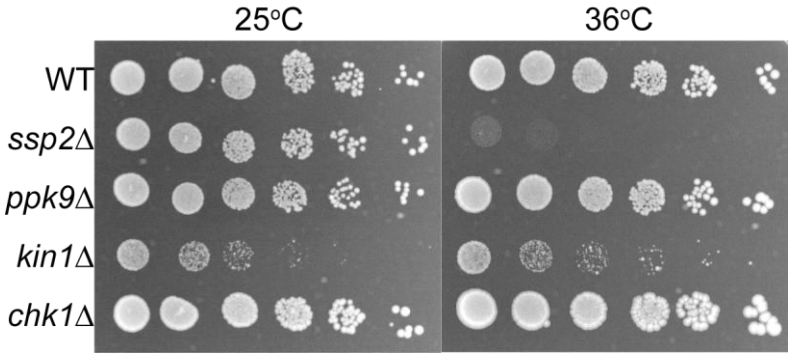


Figure 21: Spot Assays of KA Containing Kinase Null Mutants at 25°C and 36°C.
Serial dilutions of WT and single nulls all grown to the same OD_{600} were plated on YES and incubated at 25°C and 36°C. As reported previously *ssp2*Δ and *kin1*Δ both appear to be *ts*, however, *kin1*Δ *ts* phenotype appears to be "leaky".

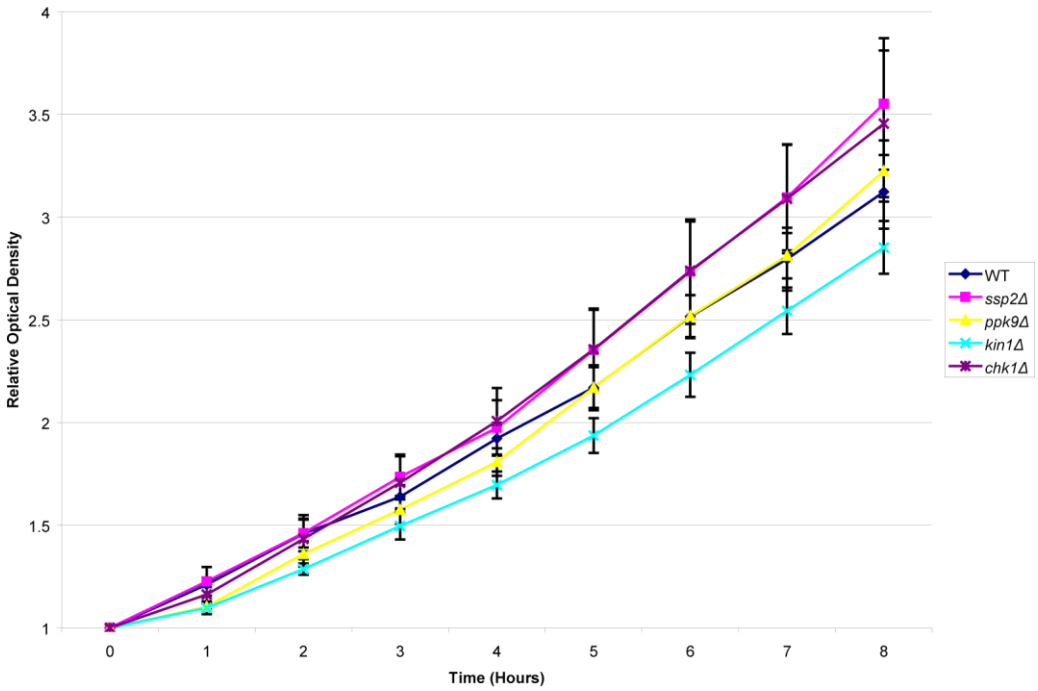


Figure 22: Growth Curve of Relative Optical Density for *ssp2*Δ, *ppk9*Δ, *kin1*Δ and *chk1*Δ Compared to WT.

Cultures were grown in YES at 25°C over night to a starting OD_{600} of at least 0.2. The OD was then measured every hour for 8 hours. The OD_{600} was then compared to OD_{600} at time 0 to give the relative OD_{600}. Each data set is an average of three independent experiments ± SE.

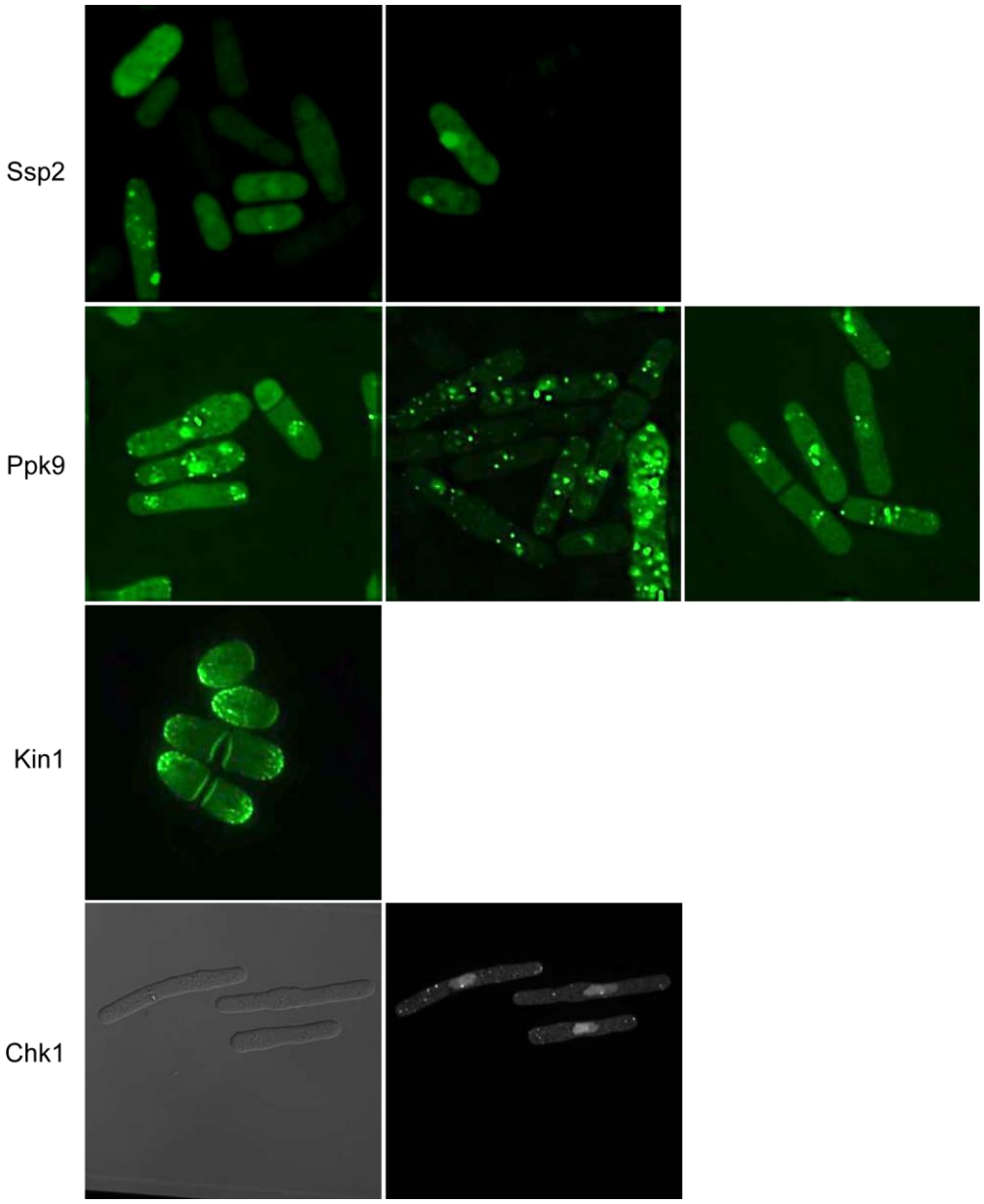


Figure 23: Localisation of Overexpressed Kinases.

Images taken the from Riken database. Open reading frames of genes were put into Gateway® vectors and used to overexpress tagged protein in *S. pombe* for all known genes. The localisation of Ssp2 is in nucleus and cytoplasm and appears as cytoplasmic spots. Ppk9 forms spots in both the cytoplasm and nucleus but is at a higher concentration in the nucleus. Kin1 is found at the cell tips and along filaments and also associates with the septum. Chk1 is found mainly in the nucleus.

database (Figure 23) show that Kin1 localises to filamentous structures at the cells tips and at the septa during division. When *kin1*Δ was crossed to *ts* mutants of essential APC/C subunits such as *cut9-665*, the resulting double mutants displayed a *kin1*Δ single mutant phenotype, but with an increase in the severity of the *ts* phenotype. However, when *kin1*Δ was crossed to the *ts* proteasome mutant *mts3.1*, the double mutant was never observed using tetrad analysis, confirming that the *kin1*Δ*mts3.1* mutant is synthetically lethal (Figure 24). This would suggest that Kin1 is involved in the Ubiquitin Proteasome System (UPS), and that disruption to both Kin1 function and the proteasome renders cells inviable due to their inability to degrade proteins.

5.2.2 *ssp2*Δ & *ppk9*Δ

Ssp2 and Ppk9 are alternative α subunits of the *S. pombe* homologue of mammalian 5' AMP-activated Protein Kinase (AMPK) (Hanyu et al. 2009). AMPK is a heterotrimer enzyme complex consisting of an α catalytic subunit, a β subunits which can bind glycogens and a γ subunit which regulates the α subunit. AMPK is activated in times of limited glucose and other stress conditions. It is responsible for stimulating the transcription of glucose repressible genes which allow cells to use other carbon sources. Although this pathway is well defined in many organisms, the mechanism in *S. pombe* is less well understood (Hedbacker & Carlson 2008). The null mutants of these kinases have no morphological defects (Figure 20); however, *ssp2*Δ is *ts* at 36^{o}C whilst *ppk9*Δ is not (Figure 21) (Matsusaka et al. 1995; Bimbó et al. 2005). Overexpression of YFP tagged protein performed by Riken shows that Ssp2 is present throughout the cell, forming cytoplasmic dots (Figure 23). Ppk9 is also present thought out the cell and formed spots; however it is more concentrated in the nucleus. Crossing to the *cut9-665* APC/C *ts* mutant showed no obvious morphological increase in phenotype for either *ssp2*Δ or *ppk9*Δ. Unlike *kin1*Δ crossing *mts3.1* proteasome *ts* with *ssp2*Δ and *ppk9*Δ resulted in viable double mutants (Figure 24).

Spot assays were performed by growing strains over night at 25^{o}C (See Material and Methods 2.3.19). These experiments showed no noticeable difference in growth of single and double mutants of *ssp2*Δ and *ppk9*Δ compared to WT on glucose, sucrose or

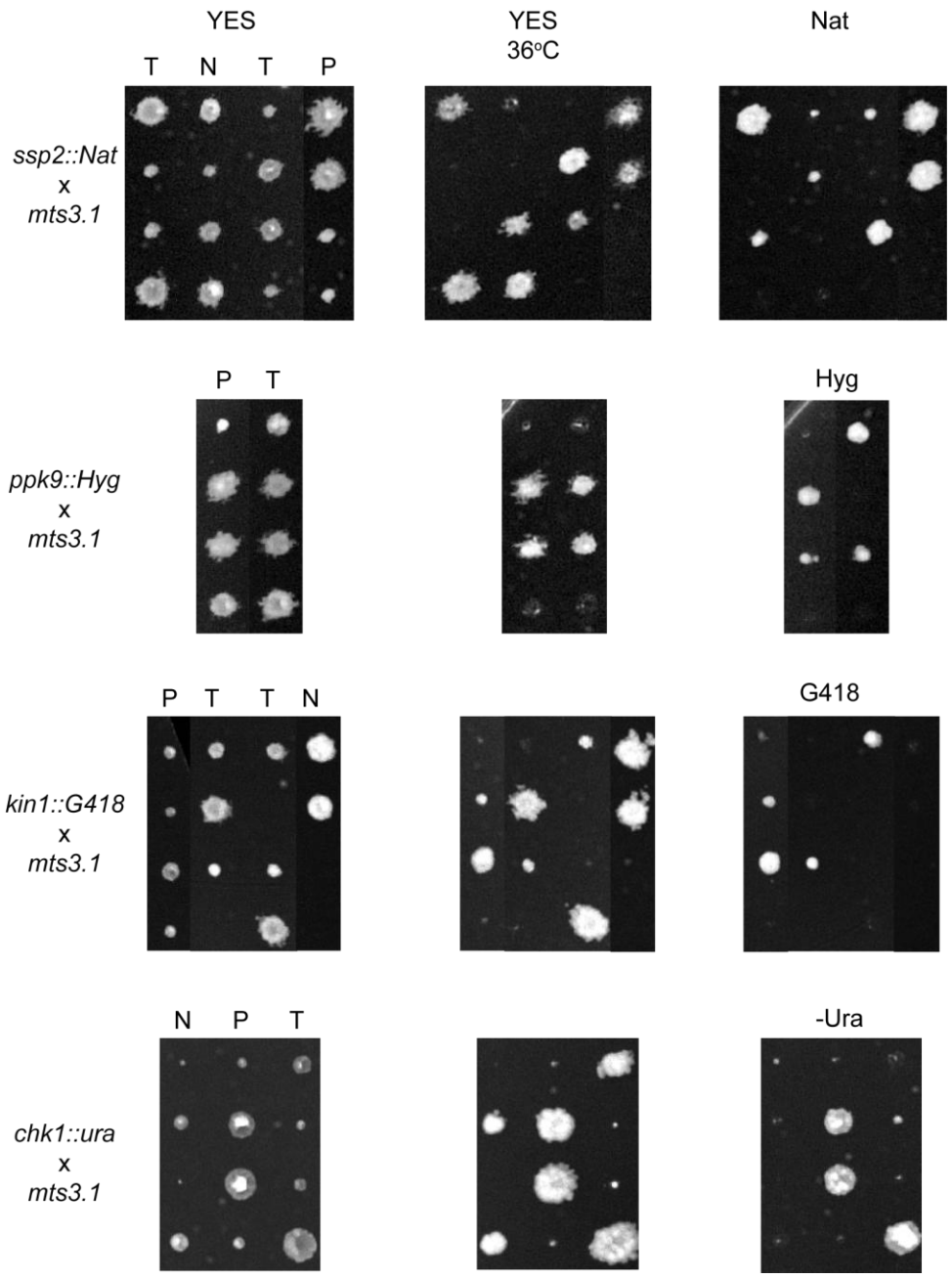


Figure 24: Tetrad Analysis of KA Containing Kinase Nulls Crossed with *mts3.1 ts*.

28 sets of spores from null kinase mutants crossed with the *mts3.1 ts* strain were separated using a Singer Micromanipulator. They were then allowed to grow to colonies on YES at 25°C then replica plated onto the appropriate selective plate and YES grown at 36°C. *ssp2*Δ, *ppk9*Δ and *chk1*Δ could form double mutants with *mts3.1 ts*. However *kin1Δ* crossed to *mts3.1 ts* only produced four viable colonies at 25°C for parental ditype (P), the tetra type (T) and non-parental ditype (N) only contained three or two viable colonies, with the double mutant absent, demonstrating that *kin1Δ mts3.1* is synthetically lethal.

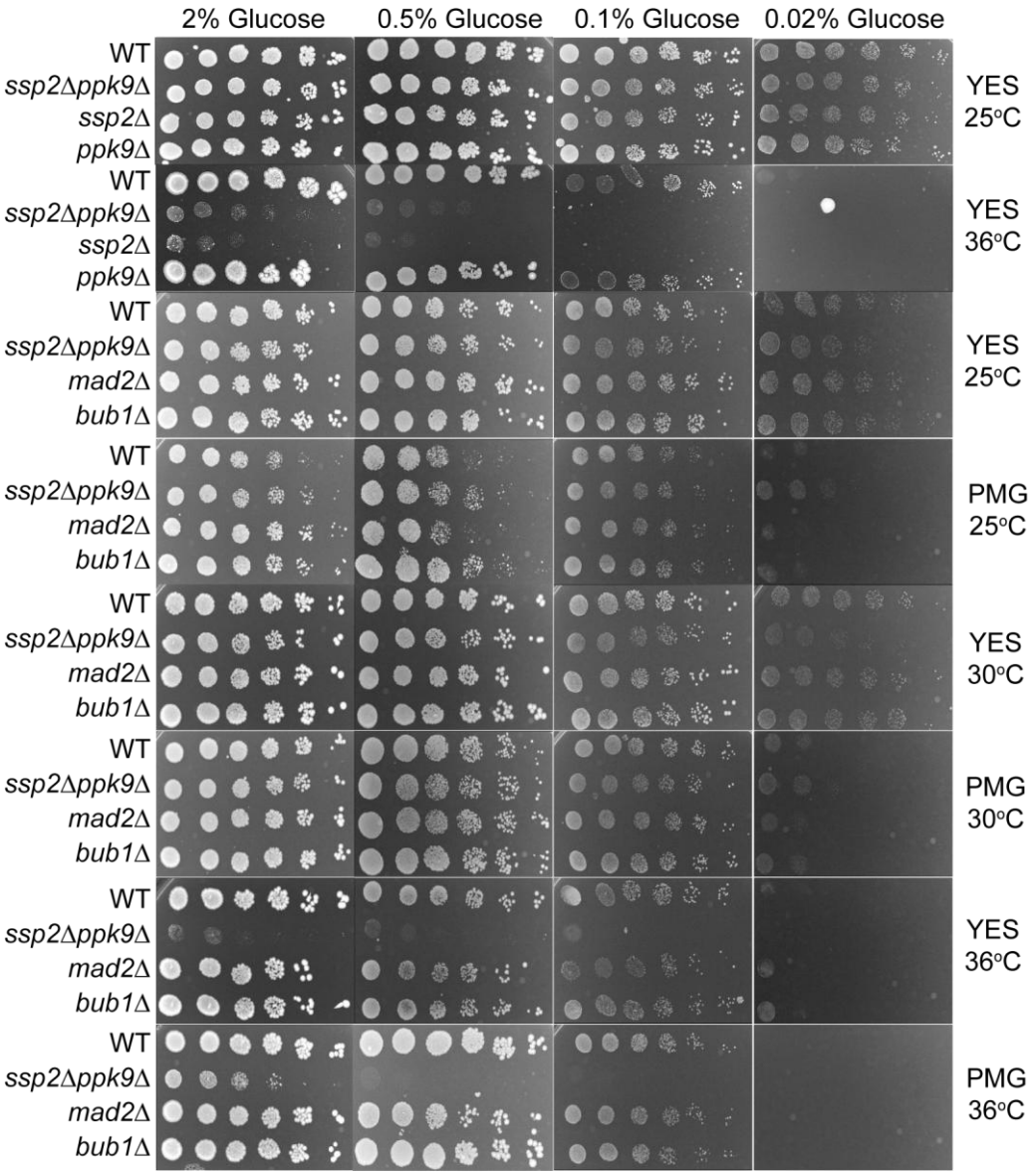


Figure 25: Growth of *ssp2*Δ, ppk9Δ, *mad2*Δ, *bub1*Δ and *ssp2*Δ*ppk9*Δ on Limited Glucose.
Cultures of test strains were grown to the same OD_{600} then equal volumes of serial dilutions were plated on YES or PMG plated with various amounts of glucose and grown at 25 °C -36°C. *ssp2*Δ*ppk9*Δ retained the *ts* phenotype of *ssp2*Δ (top two plates).

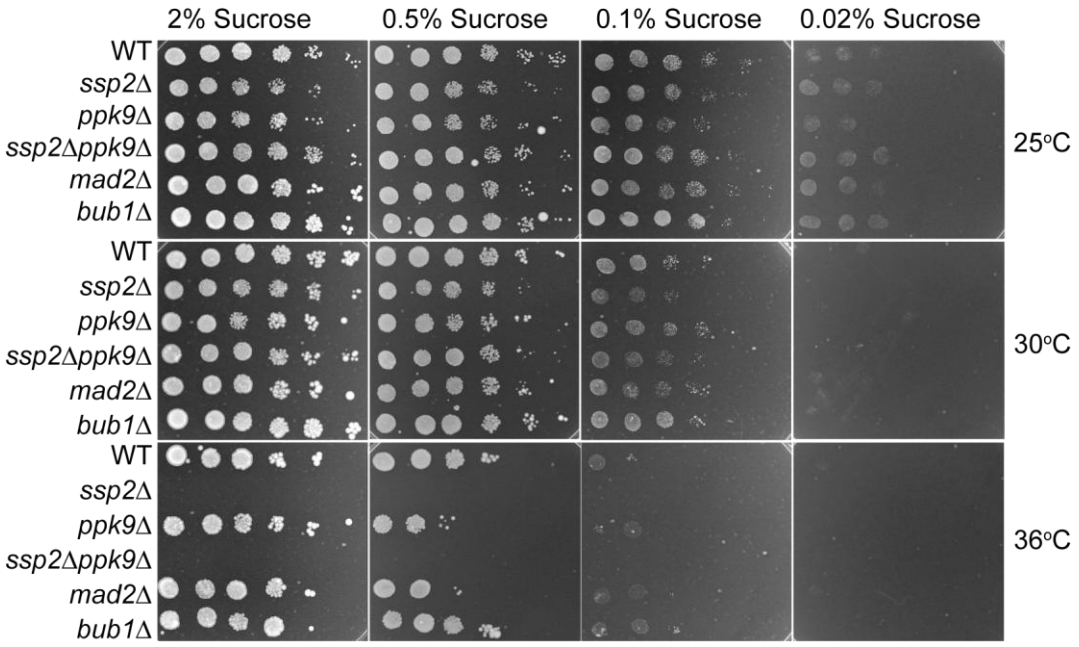


Figure 26: Growth of *ssp2*Δ, *ppk9*Δ, *ssp2*Δ*ppk9*Δ, *mad2*Δ and *bub1*Δ on Limited Sucrose.
Cultures of test strains were grown to the same OD_{600} then equal volumes of serial dilutions were plated on PMG with various amounts of the carbon source sucrose and grown at 25 °C, 30°C and 36°C.

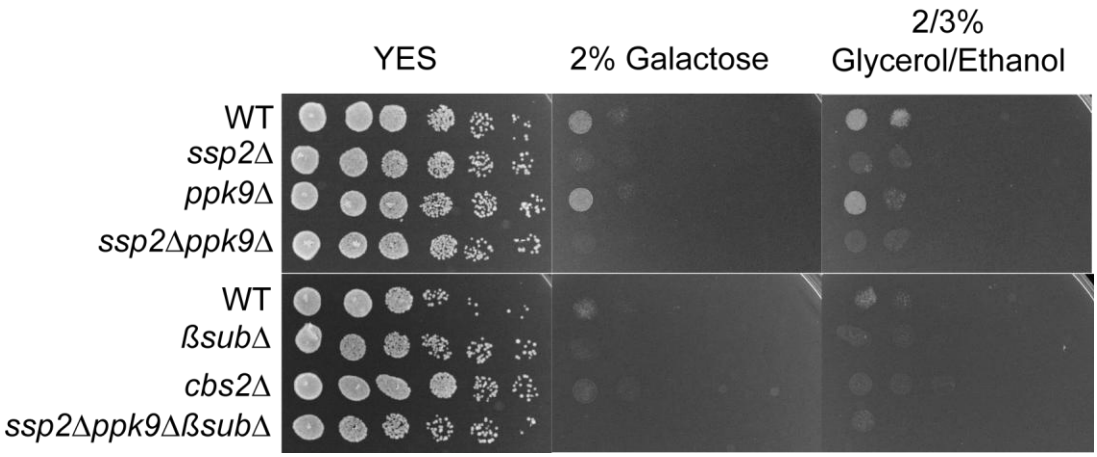


Figure 27: Growth of *ssp2*Δ, *ppk9*Δ, *cbs2*Δ, *ßsub*Δ, *ssp2*Δ*ppk9*Δ and *ssp2*Δ*ppk9*Δ *ßsub*Δ on Non-Fermentable Carbon Source.

Cultures of test strains were grown to the same OD_{600} then equal volumes of serial dilutions were plated on YES with different carbon sources and grown at 25 ^{o}C. The γ subunit, *cbs2*Δ and the β subunit SPCC1919.03.C were tested along with *ssp2*Δ and *ppk9*Δ.

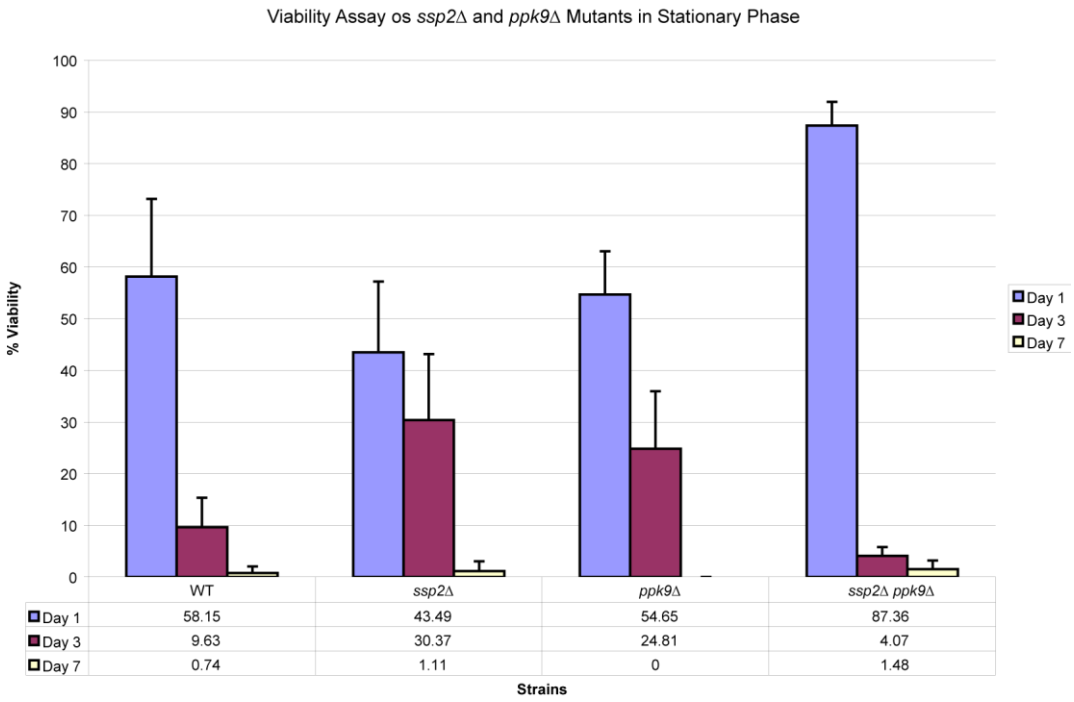


Figure 28: Viability of AMPK Mutants After Entry Into Stationary Phase.

Triplicate 20ml cultures were made using YES for each strain and grown at 25^{o}C with shaking at 180rpm. After 1, 3 and 7 days a small volume was taken and random cells were placed in a 9x10 grid using a Singer Micromanipulator (top panel). Cells were then allowed to grow for 3-5 days and the percentage viability calculated (bottom panel). No significant difference was seen between viability of WT and the single *ssp2*Δ and *ppk9*Δ mutants or double mutant, error bars are ± SE.

non fermentable carbon sources galactose and glycerol/ethanol (Figure 25, Figure 26 & Figure 27).

To question if these two proteins are important for viability after entry to stationary phase the single mutants and double mutant were grown in YES at 25^{o}C for 7 days. After 1, 3 and 7 days a small volume of culture was taken. For each culture 90 random single cells were separated into a 9x10 grid using a Singer Micromanipulator on YES plates and allowed to form colonies. The percentage of colony forming cells was calculated from two plates for each of the three replicas, but no significant difference was noted in comparison to WT (Figure 28).

5.2.3 *chk1*Δ

Chk1 is involved in the DNA damage checkpoint, and overexpressed YFP tagged Chk1 performed by Riken, shows that it localises to the nucleus (Figure 23) which is consistent with its function, in addition Chk1 also forms dots in the cytoplasm. The *chk1*Δ null mutant has no morphological defects, displays no *ts* phenotype (Figure 20 & Figure 21) and can form viable double mutants with *cut9-665* (data not shown) and *mts3.1* at permissive temperature (Figure 24). However, *chk1*Δ has increased sensitivity to DNA damaging agents such as HU and MMS (Figure 29 & Figure 30) (Walworth et al. 1993).

Sugimoto et al. (2004) demonstrated that Checkpoint DNA Synthesis 1(Cds1), a kinase that performs the same function as Chk1 at a different stage of the cell cycle (Rhind & Russell 2000), acts synergistically with Mad2. The *cds1*Δ*mad2*Δ double mutant entered mitosis early when exposed to HydroxyUrea (HU), and displayed a similar phenotype to *rad3*Δ, which has no active DNA replication checkpoint, and this double mutant has an increased sensitivity to Methyl MethaneSulfonate (MMS). This was not seen with any other double mutant of the MCC complex and would suggest that Mad2 specifically has a role in the DNA damage response pathway. To see if this was specific to Cds1 or if Chk1 also shared this apparent interaction with Mad2, a *chk1*Δ*mad2*Δ double mutant was created and the experiments performed by Sugimoto et al. (2004) repeated. First

spot assays were performed and grown on YES with 1mM or 2mM HU (Figure 29) since *chk1*Δ appeared to be more sensitive to HU than *cds1*Δ and could not grow on 5mM HU at a similar rate to WT (Sugimoto et al. 2004). The *chk1*Δ*mad2*Δ was not able to grow as well as either single mutant at 2mM HU, dminstration that Chk1 also displayed a synergistic relationship with Mad2.

To test for MMS sensitivity, cultures of all four single KA containing kinases and double mutants with *mad2*Δ were grown to mid log phase at 25^{o}C. A small volume of culture was taken and plated onto two YES plates, and MMS was then added to the culture to make a final concentration of 0.005%. The cultures were grown for 4 hours before the same volume of culture was plated onto another two YES plates. Colonies were then allowed to form by incubating at 25^{o}C and the viability of the strains, after 4hr exposure to MMS, expressed as a percentage of the colonies at time 0hr. Figure 30 shows the average results of three cultures of each strain using WT and *rad3*Δ as controls. In culture neither *chk1*Δ nor *chk1*Δ*mad2*Δ viability showed any significant deviation from WT unlike the control *rad3*Δ (p=0.0129 and 0.0067). This appears to be contradictory to the spot assays which show *chk1*Δ and *chk1*Δ*mad2*Δ can grow at 0.001% but not 0.005% MMS (Figure 31). The only strain which showed a loss in viability, apart from *rad3*Δ, was *kin1*Δ*mad2*Δ. However, statistical analysis showed that this change was not significant.

5.3 *kin1*Δ Shows an Increase in Mis-Regulation of Chromosome Segregation

APC/C is responsible for regulating the activity of the enzyme Cut2 (separase) which cleaves the cohesion complex which holds the sister chromatids together during the metaphase stage of mitosis. When APC/Cs activity is disrupted this process is then not carried out to completion which leads to unfaithful chromosome segregation. If KA containing kinases are regulating APC/Cs activity, it is possible that these mutants will also display a similar phenotype.

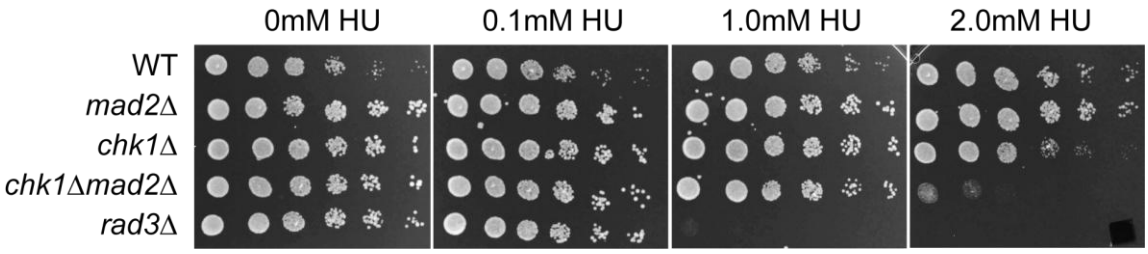


Figure 29: Effects of HU on *chk1*Δ, *mad2*Δ and *chk1*Δ*mad2*Δ.

WT, single mutants and the *chk1*Δ*mad2*Δ double mutants were grown to the same OD_{600}. Serial dilutions were then made of each culture. Equal volumes of these dilutions were then plated onto YES or YES with HU and grown at 25^{o}C. The *rad3*Δ control displayed sensitivityat 1mM/ *chk1*Δ shows some sensitivity to 2mM HU, however, *chk1*Δ*mad2*Δ displayed sever sensitivity even at 2mM HU suggesting that Chk1 and Mad2 act synergistically.

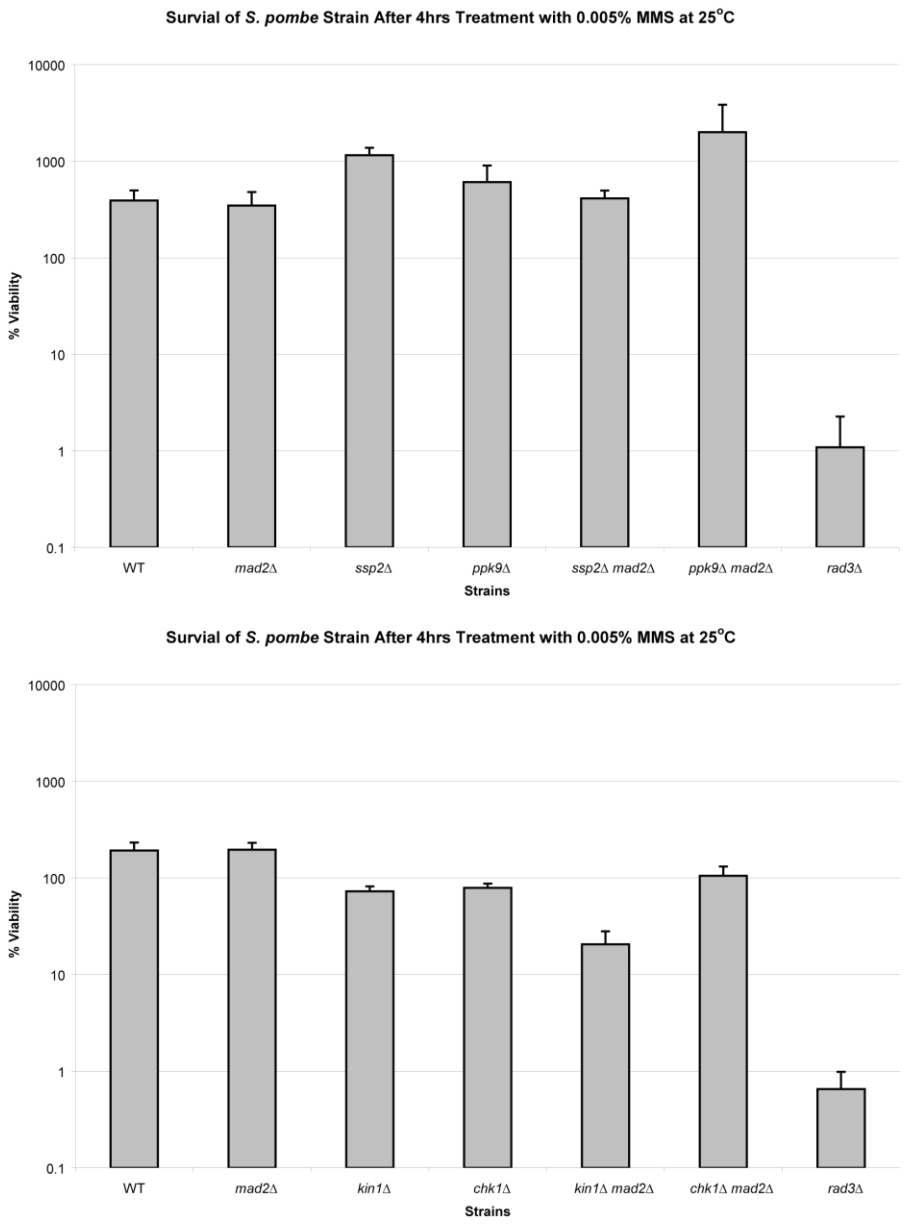


Figure 30: Survival of Mutant Strains After 4 Hours Exposure to MMS at 25°C.

WT and mutant strains were grown overnight to lag phase. A fixed volume of culture was plated onto YES plates at time 0hr. The MMS was added to the cultures to a final concentration of 0.005% and allowed to grown for 4hrs. The same volume of culture was then plated onto YES plates and colonies grown at 25°C. The viability was calculated as a percentage of colonies after 4hrs treatment compared to 0hrs. No significant decrease in viability was seen for double mutants of *mad2*Δ with the kinase nulls. *rad3*Δ was used as a positive control which gave a significant decrease in viability with P=0.0129 for the *ssp2*Δ/*ppk9*Δ experiment and P=0.0067 for *kin1*Δ/*chk1*Δ. The results shown are an average of three cultures with error bars are ± SE.

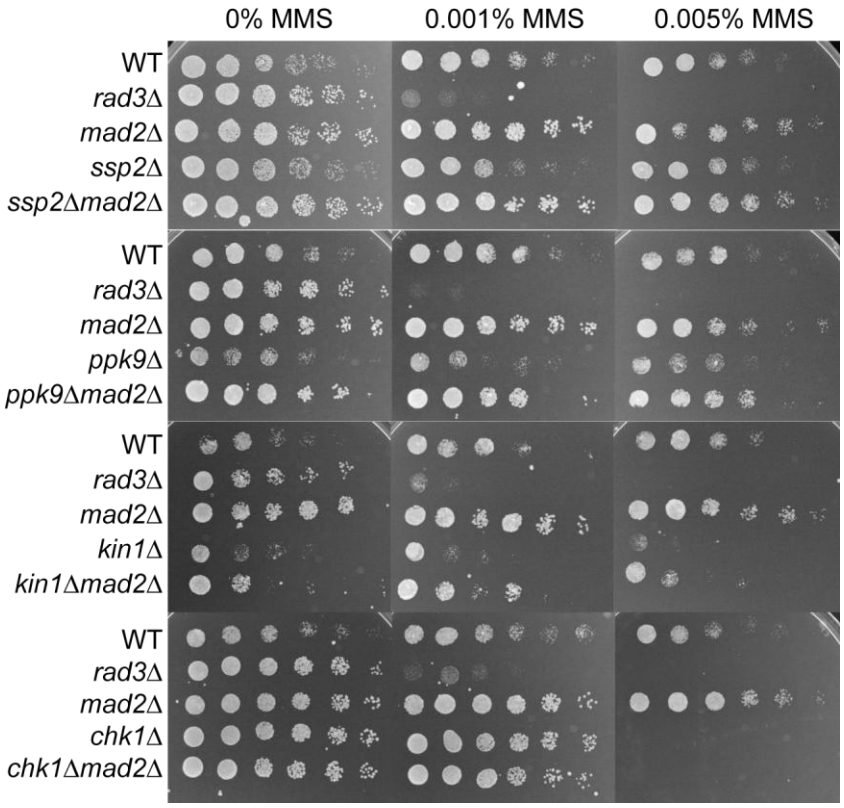


Figure 31: Growth Assay of Mutant Strains on Exposure to MMS.

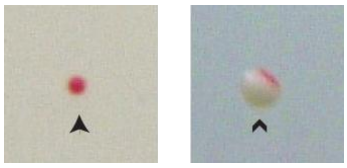
Strains used for testing viability to MMS in culture were grown to the same OD_{600} and serial dilutions made. Equal volumes were plated onto YES containing 0%, 0.001% and 0.005% MMS and grown at 25^{o}C.

A well established assay set up in *S. pombe* calculates the loss of chromosomes per cell division. The strain *ade6-210 leu1-32 ura4-DS/E [Ch16 ade6-216 m23::LEU2] h-*, used by Allshire et al. (1995) which contains an extra mini chromosome 16 containing the *ade6-216* allele in a non mutant background containing *ade6-210*. On their own either one of these alleles would render the cells unable to grow without adenine. When grown on media containing a low amount of adenine, colonies containing one of these alleles appear pink to dark red. However these two alleles complement each other, so when they are both present in the same cell, the cell displays an adenine prototrophic phenotype and will appear off white when grown on low adenine.

The *ade6-210* allele was initially crossed into the kinase mutants then the strains were crossed to the mini chromosome strain. Cultures of kinase mutants, containing the extra chromosome, were grown first in PMG-Leu-Ade to select for mutant cells carrying the mini chromosome, next the cells were plated onto PMG 1/15 Ade and grown at 30^{o}C until colonies appeared. In order to allow the red colour to deepen, plates were stored at 4^{o}C before analysis. Since every colony derives from a single cell, only colonies with more than half their sector red were counted (Figure 32 closed arrow heads, **R**) as this indicates that the chromosome loss must have occurred during the first division. Taken together with the colonies with less than half their sectors red (Figure 32 open arrow heads, **r**) and the white colonies (**W**) the percentage of chromosome loss per division can be calculated:

$$\left\{ \frac{\mathbf{R}}{(\mathbf{R}+\mathbf{W}+\mathbf{r})} \right\} \times 100 = \% \text{ chromosome loss per cell division}$$

At least 1000 colonies were counted per strain and the average percentage of chromosome loss per division calculated. These results showed the only strain which had a significant increase in the rate of chromosome loss compared to WT 0.19% was



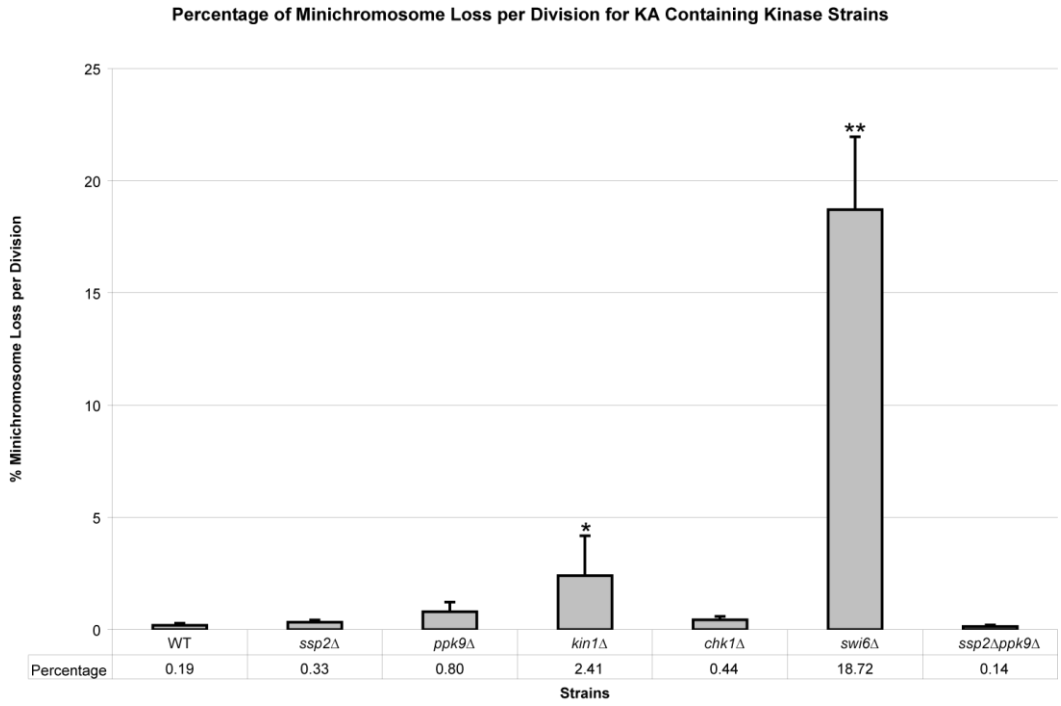


Figure 32: Measuring the Percentage of Chromosome Loss per Cell Division of KA Kinase Mutant Strains.

Null mutants carrying an *ade6-210* allele were crossed to a strain containing an artificial mini chromosome containing the *ade6-216* allele. The resulting cells were grown in PMG-leu-ade to maintain the extra chromosome and then plated out onto PMG 1/15 ade and allowed to grow. Cells which lost the mini chromosome appear red (top panel) on low ade plated. The number of colonies with at least half their sector red was counted and expressed as a percentage of total cells counted to give the percentage of chromosome losses per cell division. Only *kin1*Δ showed a significant increase compared to WT with P=0.0109 (*****) compared to the positive control *swi6*Δ which gave P<0.0001 (******), error bars are ± SE.

*kin1*Δ which has a percentage loss of 2.41% (P value of 0.0109). As a control *swi6*Δ was used which showed an 18.72% of chromosome loss per division (P value of <0.0001).

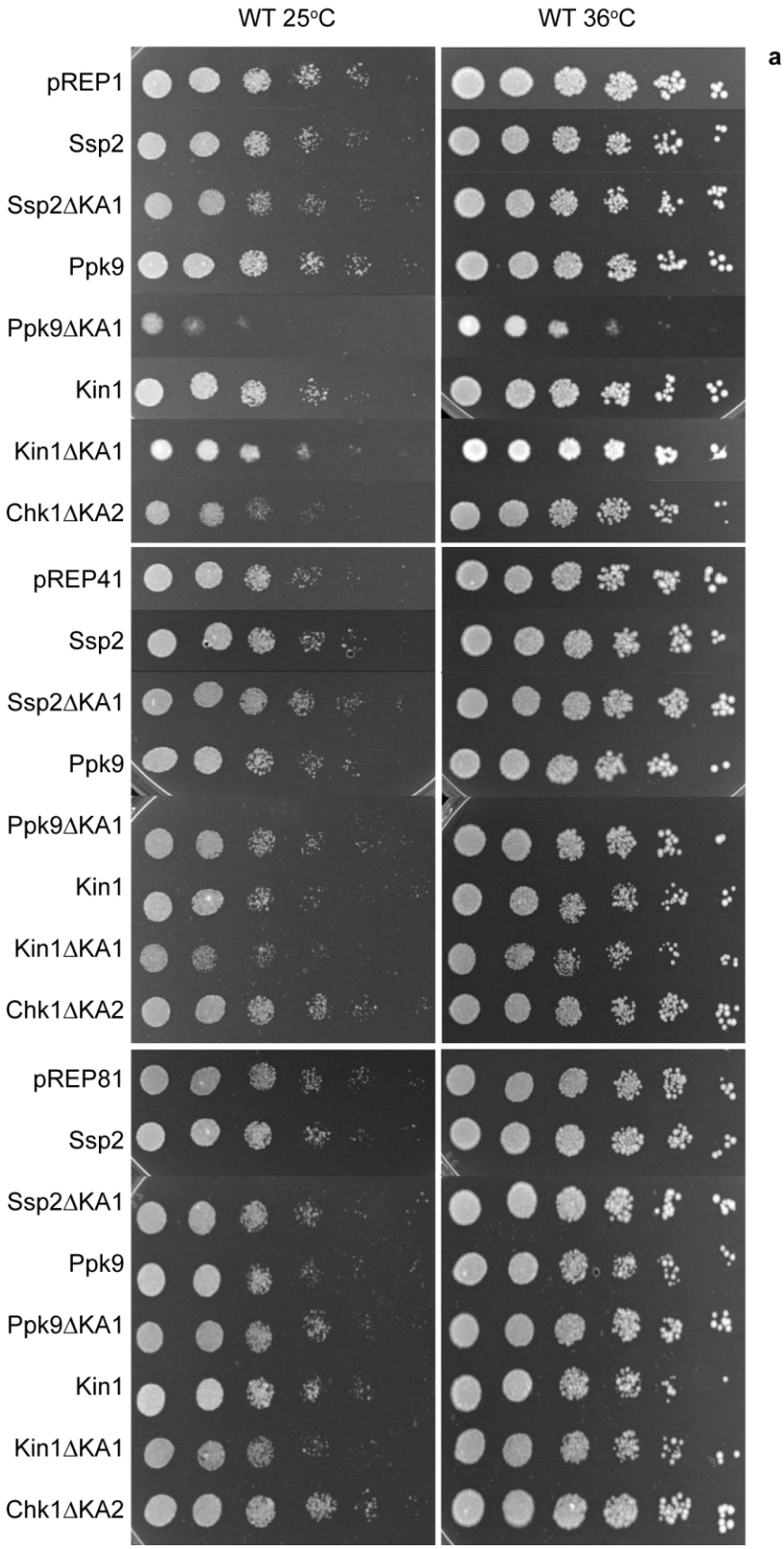
5.4 Overexpression of Kinases Does Not Rescue the *ts* Phenotype of APC/C or Proteasome Mutants

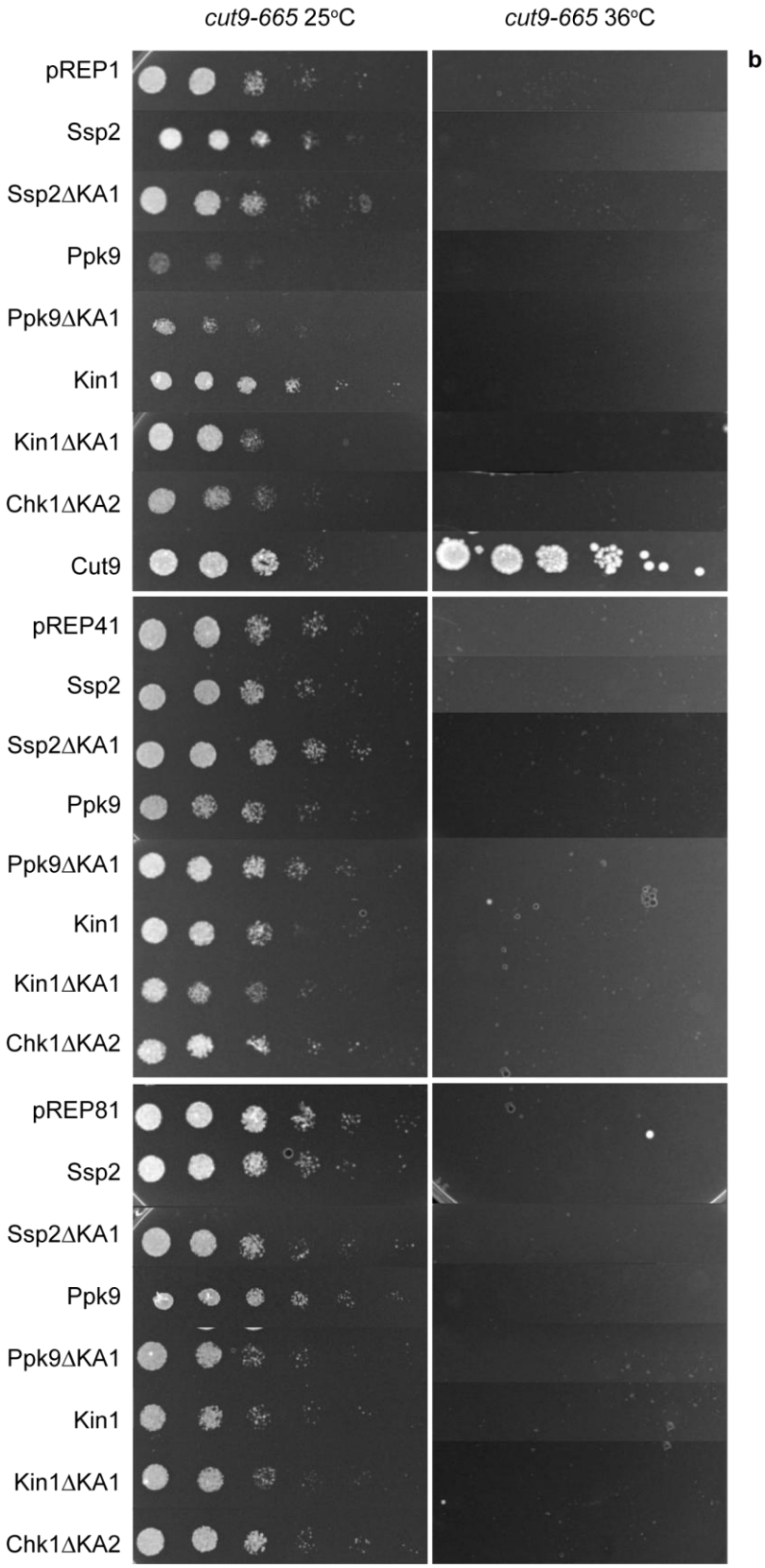
The rescue of a *ts* phenotype by overexpression of another protein can be an indication that these two proteins interact, causing stabilisation of the *ts* mutant protein (Forsburg 2001). Since the KA containing kinases interact with APC/C via Slp1, it would be reasonable to assume that the overexpression of the kinases rescue the *ts* phenotype. To test this, full length and ΔKA proteins were moved from Gateway™ entry vectors into Gateway™ converted pREP1, pREP41 and pREP81. These plasmids contain different strength *nmt* promoters: pREP1 is the strongest; pREP41 is intermediate whilst pREP81 is low strength. These were transformed into WT, *cut9-665* and *slp1-362* and spot assays were performed on PMG-Leu at 25^{o}C and 36^{o}C (Figure 33).

In the WT strain all cells grew normally under every strength *nmt* promoter (Figure 33 **a**). In the *cut9-665* strain, pREP1 expressing Ppk9 and Ppk9ΔKA maybe slightly toxic to the cells, however none of the constructs rescued the *ts* phenotype, unlike pREP1 Cut9 (Figure 33 **b**). Also no rescue was observed for the *slp1-362 ts* phenotype (Figure 33 **c**). Since a synthetic lethal phenotype was observed between *kin1*Δ and *mts3.1*, pREP1 constructs were transformed into *mts3.1* to test for rescue of the *ts* phenotype. Again, no rescue of the *ts* phenotype was observed (Figure 34).

5.5 TBZ Sensitivity

In the study by Bimbó et al. (2005), the sensitivity of all viable *S. pombe* kinase mutants to a range of different drugs and stresses were analysed. Reviewing the results they presented for the four KA containing kinase mutants, the only thing they had in common


WT 25°C
WT 36°C
a
pREP1
Ssp2
Ssp2ΔKA1
Ppk9
Ppk9ΔKA1
Kin1
Kin1ΔKA1
Chk1ΔKA2
pREP41
Ssp2
Ssp2ΔKA1
Ppk9
Ppk9ΔKA1
Kin1
Kin1ΔKA1
Chk1ΔKA2
pREP81
Ssp2
Ssp2ΔKA1
Ppk9
Ppk9ΔKA1
Kin1
Kin1ΔKA1
Chk1ΔKA2


cut9-665 25°C
cut9-665 36°C
b
pREP1
Ssp2
Ssp2ΔKA1
Ppk9
Ppk9ΔKA1
Kin1
Kin1ΔKA1
Chk1ΔKA2
Cut9
pREP41
Ssp2
Ssp2ΔKA1
Ppk9
Ppk9ΔKA1
Kin1
Kin1ΔKA1
Chk1ΔKA2
pREP81
Ssp2
Ssp2ΔKA1
Ppk9
Ppk9ΔKA1
Kin1
Kin1ΔKA1
Chk1ΔKA2

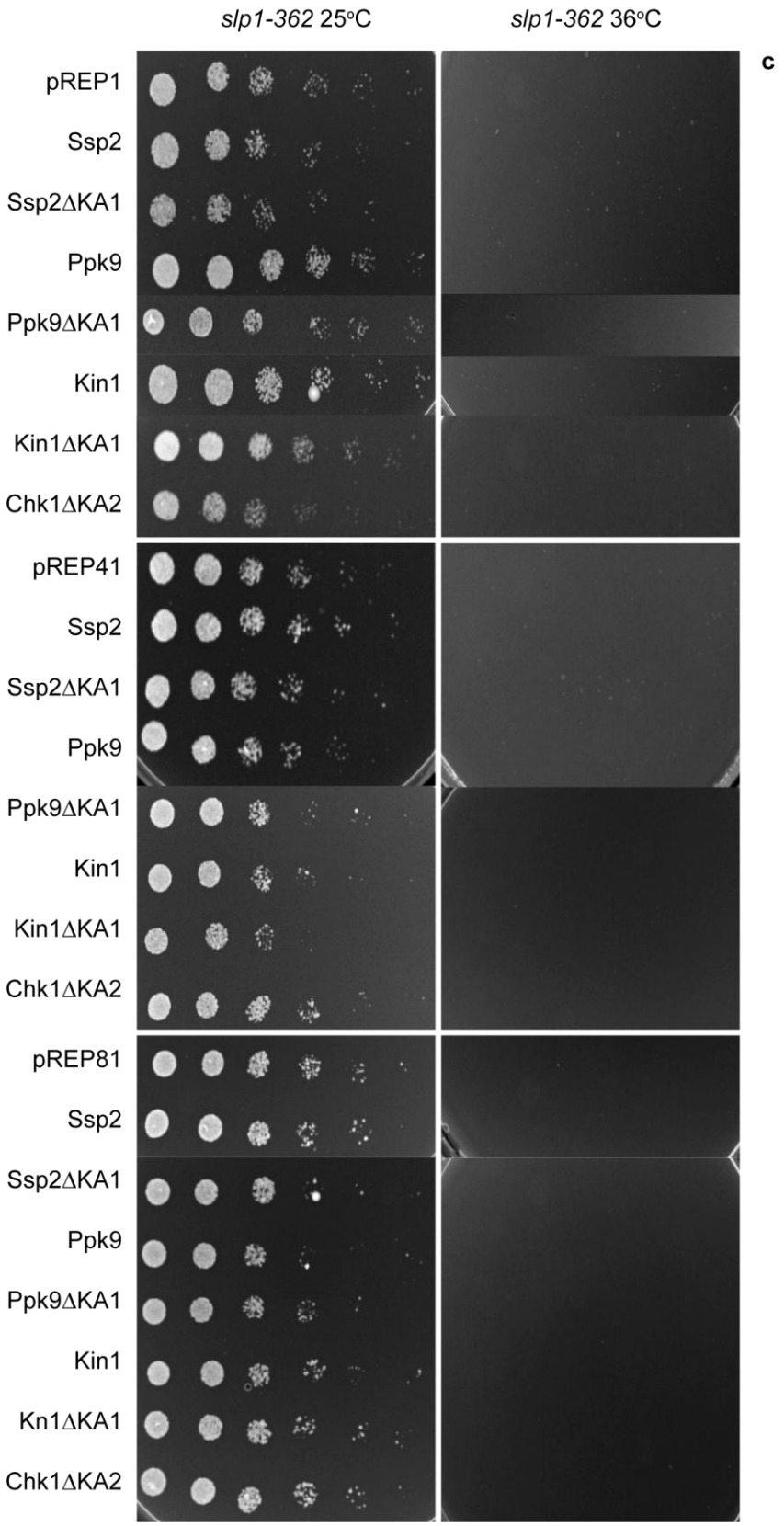

slp1-362 25°C
slp1-362 36°C
c
pREP1
Ssp2
Ssp2ΔKA1
Ppk9
Ppk9ΔKA1
Kin1
Kin1ΔKA1
Chk1ΔKA2
pREP41
Ssp2
Ssp2ΔKA1
Ppk9
Ppk9ΔKA1
Kin1
Kin1ΔKA1
Chk1ΔKA2
pREP81
Ssp2
Ssp2ΔKA1
Ppk9
Ppk9ΔKA1
Kin1
Kn1ΔKA1
Chk1ΔKA2

Figure 33: Over-Expression of Full Length and ΔKA Proteins Under the Control of Different Strength *nmt* Promoters in WT, *cut9-665* and *slp-362*.

a) WT, **b**) *cut9-665* and **c**) *slp1-362* cells transformed with pREP1, pREP41 and pREP81 constructs containing full length or ΔKA cDNA. They were grown to the same OD_{600} and serial dilutions made of each strain. Equal volumes were plated onto PMG-leu and grown at 25°C or 36°C to determine if overexpression of the KA kinases could rescue the *ts* phenotype. **a**) showed that overexpression did not affect WT cell growth at either temperature. However (**b**) and (**c**) demonstrated that they could not rescue the *ts* phenotype of *cut9-665* or *slp1-362*, unlike Cut9 which was used as a control for *cut9-665*.

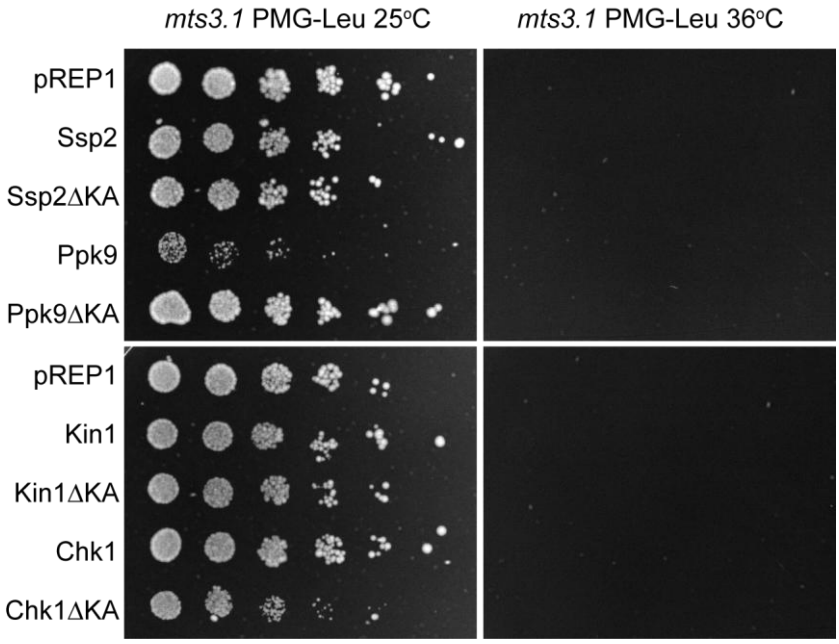


Figure 34: Over-Expression of Full Length and ΔKA Proteins Under the Control of High Strength *nmt* Promoters in *mts3.1*.

pREP1 constructs containing full length and ΔKA kinases were transformed and expressed in *mts3.1* strains to test if any could rescue the *ts* phenotype. Strains were grown to the same OD_{600} and serial dilutions made of each strain. Equal volumes were plated onto PMG-leu and grown at 25°C or 36°C

was sensitivity to the microtubule poison ThiaBendaZole (TBZ). These findings are constant with the results of this work, since the SAC is activated by TBZ (Peralta-Sastre et al. 2010). This assay was repeated with WT and the four KA kinase mutants transformed with pREP1 containing full length or ΔKA. Spot assays were performed with YES media containing 0µg/ml, 5µg/ml and 10µg/ml TBZ (Figure 35). Again, the overexpression of Ppk9 and Ppk9ΔKA appears to be toxic to WT cells, whilst *ppk9*Δ transformed with Ppk9 showed a lower growth rate on 5µg/ml TBZ compared to *ppk9*Δ transformed with empty pREP1. Of the four mutants only *kin1*Δ had any noticeable sensitivity to TBZ, which contradicts the findings of Bimbó et al. (2005), which suggested that *ssp2*Δ, *ppk9*Δ and *chk1*Δ only displayed mild sensitivity to TBZ. The expression of full length Kin1 did not completely rescue the phenotype, but Kin1ΔKA appeared to decrease sensitivity. This may be due to the overexpression of the protein using the pREP1 strong promoter. La Carbona et al. (2004) demonstrated that the strong overexpression of Kin1 can give rise to a *kin1*Δ phenotype.

It has also been demonstrated that *mad2*Δ shows TBZ sensitivity (Tange & Niwa 2007). The full length and ΔKA constructs for the four kinases were transformed into *mad2*Δ::ura and expressed using the pREP41 plasmid to test if overexpression had any effect on TBZ sensitivity (Figure 36). These results showed that on plates lacking TBZ, all of the strains appeared to grow at a similar rate. Exposure to 5µg/ml TBZ showed that *mad2*Δ::ura expressing the ΔKA constructs of Ssp2, Ppk9 and Chk1 grew better than full length or pREP41 control. Kin1 did not share this phenotype; in fact the ΔKA construct grew poorly compared to the full length. Again this may be due to overexpression causing a dominant negative effect.

5.6 Discussion

Of the 106 kinases encoded in *S. pombe*, only three have a KA1 and one has a proposed KA2 domain. Single null mutants of *ssp2*Δ, *ppk9*Δ, *kin1*Δ and *chk1*Δ have been made and three have been published (Walworth et al. 1993; Matsusaka et al. 1995; La Carbona

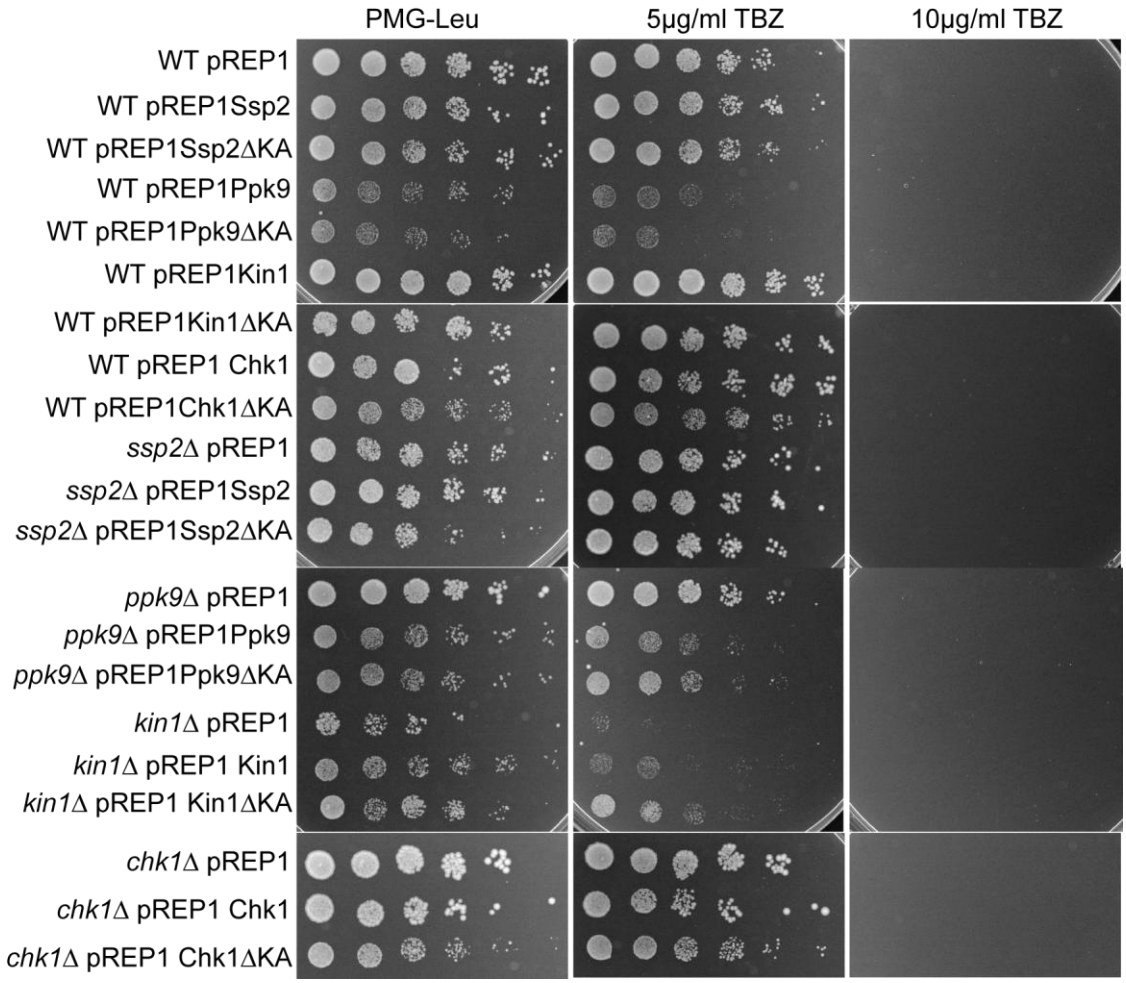


Figure 35: Effects of TBZ on WT and Kinase Mutants Over-Expressing Full Length and ΔKA Protein.

WT and kinase null strains were transformed with empty pREP1 of pREP1 containing full length and ΔKA constructs. Cultures were grown to the same OD_{600}. Then equal volumes of serial dilutions were plated onto PMG-leu containing 0, 5 or 10µg/ml TBZ.

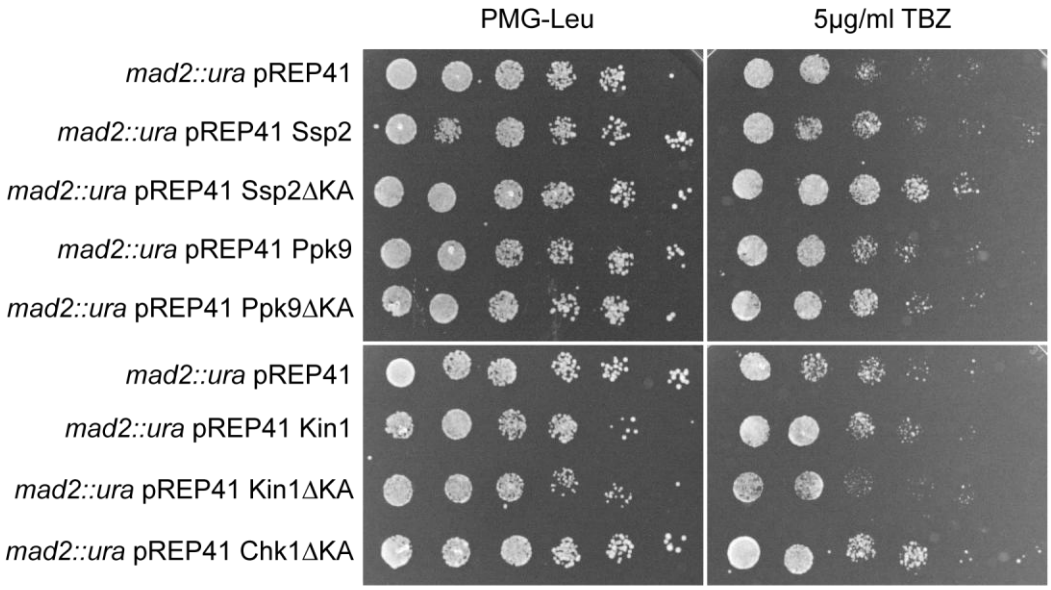


Figure 36: Effects of TBZ on *mad2Δ* Mutants Over-Expressing Full Length and ΔKA Protein. *mad2Δ* null strain was transformed with empty pREP41 or pREP41 containing full length and ΔKA constructs. Cultures were grown to the same OD_{600} the equal volumes of serial dilutions were plated onto PMG-leu containing 0µg/ml or 5µg/ml TBZ.

et al. 2004). All of these mutants are viable, as is every combination of double, triple and even the quadruple mutant. Chapter 3 demonstrated that the KA domain can bind APC/C and the results of Chapter 4 would suggest that the reason for this binding is to regulate APC/Cs activity. These genetic results show that this function is not essential for cell survival.

As the KA domains have been shown to bind to APC/C, the null mutants were crossed to the APC/C *ts* mutant *cut9-665* to see if the double mutants displayed any additional phenotype. Nothing was seen for *cut9-665*, however, when the null mutants were crossed to the proteasome *mts3.1 ts* strain, the *kin1*Δ *mts3.1*double mutant was synthetically lethal at 25^{o}C. This suggests that Kin1 has a role in protein degradation via the proteasome and that both of these proteins need to be active for efficient degradation, otherwise the cell cannot survive. This was not seen for the other four kinases. It is known that if overexpression of one protein rescues the *ts* phenotype of another protein mutant strain, this can be an indication that the two proteins interact, and that the overexpressed protein can stabilise the *ts* mutant protein (Forsburg 2001). To look for evidence of interaction between the KA containing kinases and UPS, full length and ΔKA protein were overexpressed in *cut9-665*, *slp1-362* or *mts3.1* mutant strains. None of the constructs tested could rescue the *ts* phenotype of these mutants.

Controlling APC/Cs activity is essential for regulating the segregation of chromosomes. A disruption to the normal regulation of APC/C activity would allow cells to enter anaphase before all the kinetochores have been bound by microtubules and therefore can lead to mis-segregation of chromosomes. The experiments using the extra chromosome Ch16 strains showed that the *kin1*Δ displayed an increase in chromosome loss not seen in the other mutants, again proving a link between Kin1 and the UPS. Although none of the other mutants showed an increase in mis-regulating chromosome segregation it would be interesting to see what effect the quadruple mutant has on the rate of chromosome loss.

Despite Bimbó et al. (2005) reporting that all four null mutants has some degree of TBZ sensitivity; this study demonstrated that *kin1*Δ was the only strain which had any noticeable sensitivity. Unexpectedly, expressing full length Kin1 did not rescue this phenotype; this may be due to the protein being too highly overexpressed in pREP1. It has been previously reported that Kin1 is expressed at low levels in the cell and that overexpression can mimic the null phenotype (La Carbona et al. 2004). *mad2*Δ has also been shown to be sensitive to TBZ because the SAC has been deactivated. Interestingly the ΔKA constructs of Ssp2, Ppk9 and Chk1 seemed to partially rescue the *mad2*Δ sensitivity to TBZ. Although ΔKA constructs cannot bind APC/C, our hypothesis that the KA domain also acts as an auto-inhibiting domain for the N-terminal kinase domain would also mean that these protein have increased kinase activity.

An interesting observation is that *ssp*2Δ is *ts* but *ppk*9Δ is not. Ssp2 and Ppk9 have not been well studied in fission yeast, so it is not known how they differ when they are part of the AMPK complex, but this difference in phenotype would suggest they may have different targets and may perform different functions.

Cds1 is the fission yeast homologue of Chk2 a similar protein to Chk1. Cds1 is activated in the same manner as Chk1 and causes a similar delay in the cell cycle. However the difference between the two kinases is that Cds1 is activated at the S phase replication checkpoint, where as Chk1 is activate at the G_2-M phase boundary. Studies have shown in the absence of Cds1, Chk1 can also activate this pathway, demonstrating that the two proteins have some overlap in function (Rhind & Russell 2000; Paparatto et al. 2009). Cds1 has been shown to phosphorylate the APC/C co-activator Ste9 during S phase which causes inactivation of APC/C. This in turn prevents the ubiquitination and degradation of factors whose presences inhibits the onset of G_2 (Chu et al. 2009). Sugimoto et al. (2004) showed that Mad2 and Cds1 act in a synergistic manner. A double null mutant of *mad2*Δ*cds1*Δ acted similar to *rad3*Δ when exposed to HU and entered mitosis early producing short cells. Mad2 was the only MCC component to produce this phenotype. They also suggest that a similar mechanism may exist for Chk1. To test this, the MMS experiments performed by Sugimoto et al. (2004) were repeated

with all the kinase mutants. Contrary to expectation *chk1*Δ and *chk1*Δ*mad2*Δ did not show a decrease in viability. The only mutant that had any decrease in viability after 4 hours of 0.005% MMS exposure was *kin1*Δ*mad2*Δ, although this was not statistically significant. However growth assays showed that *chk1*Δ and *chk1*Δ*mad2*Δ could not grow on plates containing 0.005% MMS. One explanation is that a 4 hour exposure to 0.005% MMS may be enough to cause loss of viability in *cds1*Δ *mad2*Δ but not in *chk1*Δ*mad2*Δ.

Although these experiments did not identify a phenotype that all these four kinases share, the *kin1*Δ often displays a phenotype that the other three null mutants did not. The KA1 domain of Kin1 is the best defined, and in addition, Kin1 is the only protein that is expressed under normal conditions whereas the remaining three kinases are only expressed under certain conditions. Chk1 is activated during DNA damage, whilst Ssp2 and Ppk9 are part of the AMPK complex which is activated at times of limited glucose and cellular stress. It may be the case that phenotypes will only present themselves when the cells are exposed to conditions in which these proteins would become activated. Therefore, under the conditions used during this study, a phenotype may not be displayed for these mutants.

Chapter 6
Function of Rhp23 UBA Domains

6.1 Introduction

The next stage in the UPS, after ubiquitination by E3s such as APC/C, is for the substrates to be recognised and transported to the proteasome. This can be done in a variety of ways. Some E3 ligases can interact with the proteasome (Xie & Varshavsky 2000; Fu et al. 2010), others utilise proteins, know as shuttle factors, which in *S. pombe* consist of Pus1 (budding yeast Rpn10), Rhp23 (Rad23) and Dph1 (Dsk2) which chaperone substrates to the proteasome. These proteins have domains which recognise and bind the proteasome and also possess the ability to recognise the ubiquitin chains tagging substrates using ubiquitin binding domains. At their N-terminus Pus1 has a von Willebrand factor A (vWA) domain whilst Rhp23 and Dph1 have a UBiquitin Like (Ubl) domain which they use to interact with the proteasome. At the C-terminus, Pus1 contains a Ubiquitin Interacting Motif (UIM) forms a single alpha helix able to bind K48 and K63 chains whilst the UBiquitin Associated (UBA) domains found in Rhp23 and Dph1 form three consecutive alpha helices which are also capable of binding either chain type (Seeger et al. 2003); Hartmann-Petersen et al. 2003; Schreiner et al. 2008). After successful delivery to the proteasome, the substrates are deubiquinated and unfolded in order to be loaded into the proteasome and degraded.

It is interesting to note that Pus1 is also a subunit of the proteasome and can act as a proteasome based receptor for ubiquitin binding along with two other subunits, Rpn13a and Rpn13b which recognise ubiquitin via a Pleckstrin-like Receptor for Ubiquitin (Pru) domain (Husnjak et al. 2008). In budding and fission yeast, it has been demonstrated that the remaining shuttle factors use their Ubl domains to interact with homologues of *S. pombe* Mts4 (Rpn1) and Rpn2 (Rpn2) to recognise and interact with the proteasome (Elsasser et al. 2002; Seeger et al. 2003; Schreiner et al. 2008). The Ubl domain possesses a similar structure to ubiquitin and as a result in some cases, they too are also capable of interacting with ubiquitin binding domains (Ryu et al. 2003). This has led to

the hypothesis that after delivery of substrates to the proteasome the UBA domains then bind to the Ubl domain causing the protein to dissociate (Hai Rao & Sastry 2002). As shown in Chapter 3, the shuttle factors have also been shown to bind E3 ligases such as APC/C (Seeger et al. 2003; Kim et al. 2004) which allow them to bind to substrates whilst still attached to a ligase.

Genetic experiments show that shuttle factors display a certain amount of redundancy in fission and budding yeast since null mutants in any one of these three genes result in viable cells. However, genetic assays show that double mutants of shuttle factors display a more severe phenotype in *S. pombe*. Crossing *rhp23*Δ to *pus1*Δ results in a double mutant that displays a growth defect at 25^{o}C and becomes inviable when grown at 36^{o}C due to the extra stress put on the UPS under these conditions. The double mutants of *dph1*Δ*pus1*Δ and *dph1*Δ*rhp23*Δ do not result in the same phenotype indicating that Dph1 is not as important as Pus1 and Rhp23. However, the triple mutant of *rhp23*Δ*pus1*Δ*dph1*Δ is synthetically lethal resulting in spores which cannot form cells at any temperature indicating that import of substrates to the proteasome via the shuttle factors is critical for cell survival (Wilkinson et al. 2001).

Rhp23 is of particular interest because, unlike most ubiquitin binding proteins, it contains not one but two domains capable of binding ubiquitin, an interior UBA1 and a UBA2 at its C-terminus. This structure is conserved to humans, which would suggest that both UBA domains are important for Rhp23 to carry out its function. Studies show that both minimal domains are capable of binding ubiquitin which has lead to the question of why Rhp23 requires two UBA domains. Do they have different functions and, if so, what are their individual roles? Analysis of the protein showed that these domains differ greatly in sequence but have the same structure (Mueller & Feigon 2002).

Previous work in this group studied the function of the two domains before the structure had been solved. To do this, a conserved lysine residue found in all UBA domains was mutated to proline which disrupted ubiquitin binding. However, this also resulted in

major changes to the structure of the protein which may be responsible for any observed change in function rather that the ubiquitin binding (Wilkinson et al. 2001). In order to determine if both UBA domains are involved in the Rhp23 ability to recognise ubiquitin, point mutations were made that are predicted to abolish ubiquitin binding without changing the structure of the protein. These proteins were used to test the ability of each domain to bind K48 and K63 chains. Domain swap chimeric proteins were made to further dissect the ubiquitin binding affinities of each UBA and to help determine if the position of the domain can affect its binding affinity. Finally the constructs were then used to rescue the *rhp23*Δ*pus1*Δ*dph1*Δ phenotype to test the UBA function *in vivo* for the first time in *S. pombe*.

6.2 Design of UBA Mutant and Domain Swap Constructs

Previous studies have looked at the role of the UBA domains in Rhp23 by making point mutations in the domains to substitute a residue with proline, which destroyed the helical structure of the protein (Wilkinson et al. 2001). In order to improve on these studies, it was important to make point mutations which would disrupt the ability of the domains to bind ubiquitin but maintain the integrity of the protein structure. Our collaborator Jane Endicott, a structural biologist from Oxford, used the structure of the UBA domain published by Ryu et al. (2003) and identified suitable candidate residues. From this structure, she predicted that M157 and L183 in UBA1 and the corresponding residues in UBA2, L332 and F358 should be important for ubiquitin binding. These residues were mutated to alanine by site directed mutagenesis of full length *rhp23+* to give full length *rhp23* with either the UBA1 domain mutated (*rhp23 M157AL183A*), UBA2 domain mutated (*rhp23 L332AF358A*) or both domains mutated (*rhp23 M157AL183A L332AF358A*).

These constructs were used to subclone full length protein into pREP to express protein in *S. pombe* under the control of *nmt* promoters. Full length and isolated UBA1

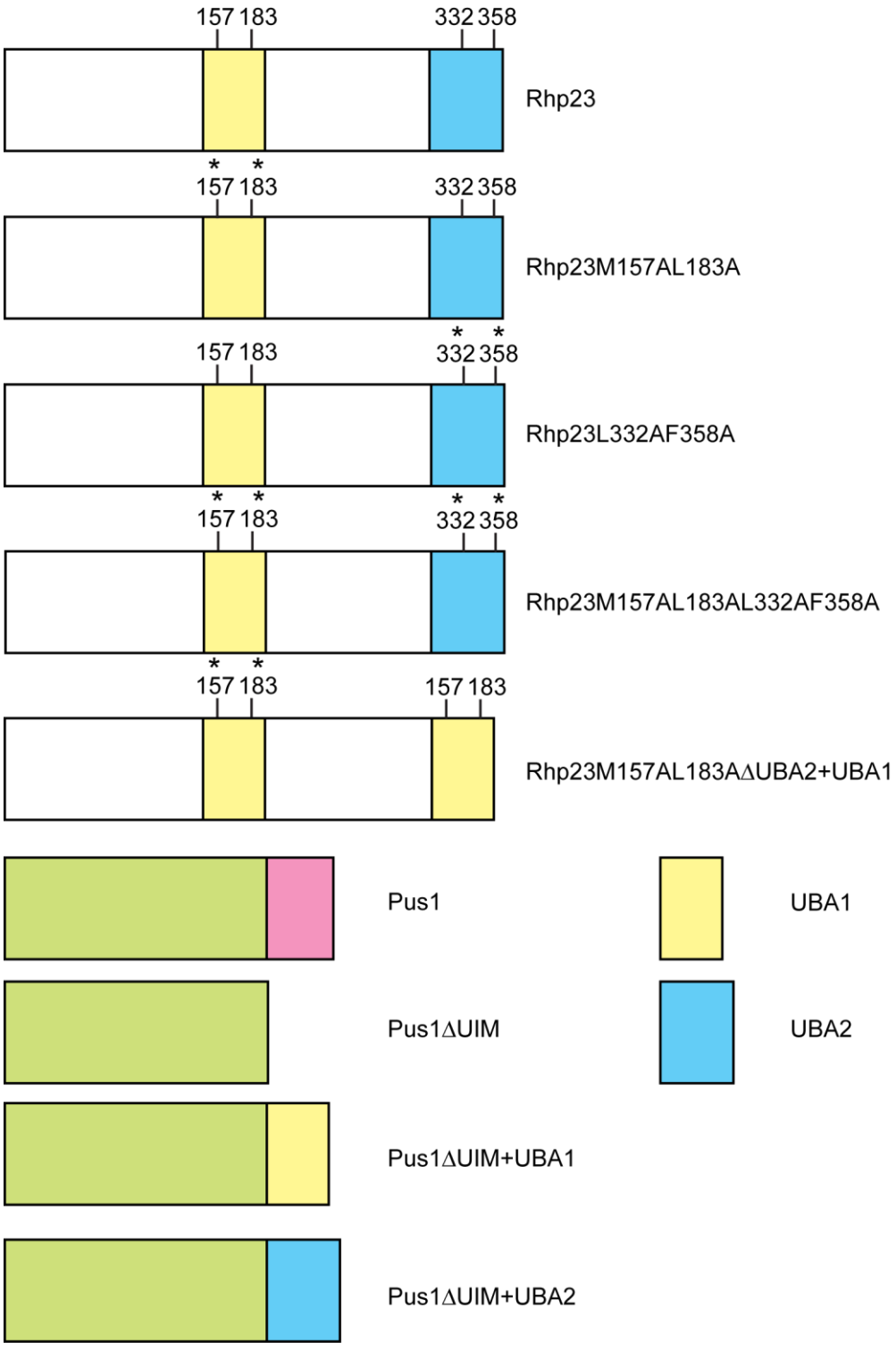


Figure 37: Constructs Made in GST and *nmt* Expression Vectors.

Full length *rhp23+* was used to carry out site directed mutagenesis on two residues in the UBA1 and

UBA2 domains. UBA1 domain was taken to be amino acids 146-190 of Rhp23, whilst UBA2 was 314-368. Homologous PCR was used to replace the UBA2 domain of *rhp23+* with the UBA1. The same technique was used to replace the UIM of *pus1+*, residues 196-243, with either a UBA1 or UBA2 domain and cloned into pGEX 6P1, pREP1, pREP41 and pREP81.

consisting of amino acids 146-190 and UBA2 residues 314-368 were cloned into pGEX 6P1 to express GST protein in order to study the domains (Figure 37). After expression of GST fusion proteins in BL21 cells, the protein was coupled to Glutathione Sepharose™ 4B beads and analysed by separating on 10% SDS PAGE gels and staining with Coomassie. This produced single bands of proteins that ran at the expected size (Figure 38 & Figure 39). NMR analysis by Jane Endicott's group confirmed that the mutations in the full length Rhp23 protein did not change the structure of the protein (data not shown).

Domains swaps were performed using homologous PCR to produce constructs with no additional enzyme sites at the junctions. Briefly, primers were designed that would allow the N-terminus and C-terminus of the chimeric protein to be cloned into pGEX 6P1. Reverse primers for the N-terminal fragment were designed to contain the beginning region of the C-terminal fragment, whilst the forward primer of the C-terminal fragment contained the end region of the N-terminal fragment. Two separate PCRs were performed to amplify the regions to be joined which were then both used as templates. The regions of homology allowed the fragments to pair up so that the new domain swap gene could be amplified and cloned into a vector.

For the Pus1ΔUIM amino acids 1-195 of Pus1 were used and the UBA domains described above were added to the C-terminus to produce the chimeric proteins. For the Rhp23M157AL183AΔUBA2+UBA1 amino acids 1-313 of Rhp23 were used which contained M157A and L183A mutations, then amino acids 146-190 of UBA1 were added to the end (see Figure 37). These GST tagged proteins were expressed and analysed as stated above to show they produced a single product of the correct size (Figure 40 & Figure 41).

6.3 Point Mutations in Isolated UBA Domains Inhibits Ubiquitin Binding

To test if these mutations affected ubiquitin binding, GST tagged full length Pus1 and Rhp23 were expressed and coupled onto Glutathione Sepharose™ 4B beads along with WT and mutated UBA1 and UBA2 domains. These proteins were incubated with K48 or K63 chains as described in 2.6.18 and excess unbound chains removed by washing. After separating on 10% SDS PAGE gels the GST fusion proteins were visualised by Coomassie staining to show equal loading of GST input protein (Figure 38 top panel). Western blot analysis with anti ubiquitin antibody (Figure 38 bottom panel) shows that the GST negative control did not bind chains, whilst the Pus1 and Rhp23 positive controls bound both types of ubiquitin chains. When tested, the isolated UBA1 and UBA2 domains of Rhp23 were able to bind the chains but not at the same level as Rhp23. However, when the domains were mutated they could no longer bind either chain type thereby confirming that the two point mutations abolished the domains ability to recognise ubiquitin chains without changing the structure.

6.4 Point Mutations in Rhp23 UBA1 Domain Inhibit or Decrease Ubiquitin Binding but Mutations in UBA2 Allow Binding.

To analyse the role of these domains set within their natural context of the full length protein, a non-mutated full length Rhp23 along with the following mutated full length versions were created: in UBA1 M157A and L183A, in UBA2 L332A and F358A and for both domains (UBA1 and UBA2) M157A, L183A, L332A and F358A. To test these mutant proteins for their ability to bind ubiquitin K48 and K63 chains, the same experiment was carried out as described for the isolated domains. These GST fusion proteins were of the correct size and appeared to be equally loaded (Figure 39 top panel). Again the Western blot shows that the GST control could not bind chains, whilst the positive control of Pus1 and Rhp23 WT proteins could bind both types of chains.

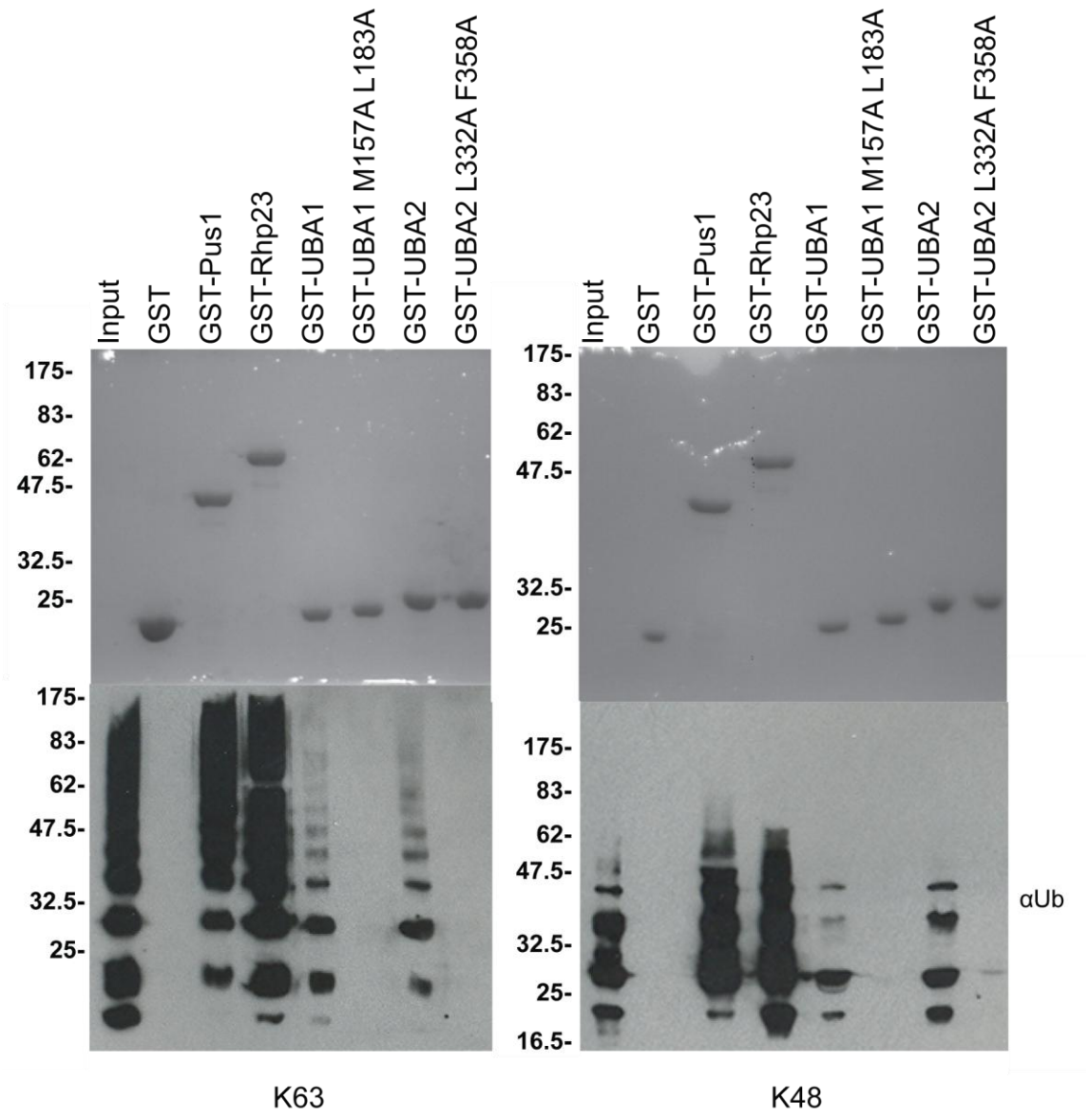


Figure 38: Ubiquitin Binding Assays for Isolated UBA Domains.

GST tagged proteins were expressed in bacteria and coupled to Glutathione Sepharose™ 4B beads. After incubating with either K63 or K48 ubiquitin chains, unbound protein was removed by washing. Proteins were then separated on 10% SDS PAGE and either Coomassie stained (top panel) or transferred onto membranes to be probed with anti ubiquitin antibody (bottom panel). The stained SDS PAGE gels of input GST fusion protein shows equal loading for full length Pus1, Rhp23 as well as WT and mutated UBA domains. The bottom panel show that full length protein and WT domains can bind both types of chains, whilst the point mutations rendered the domains unable to bind either chain type.

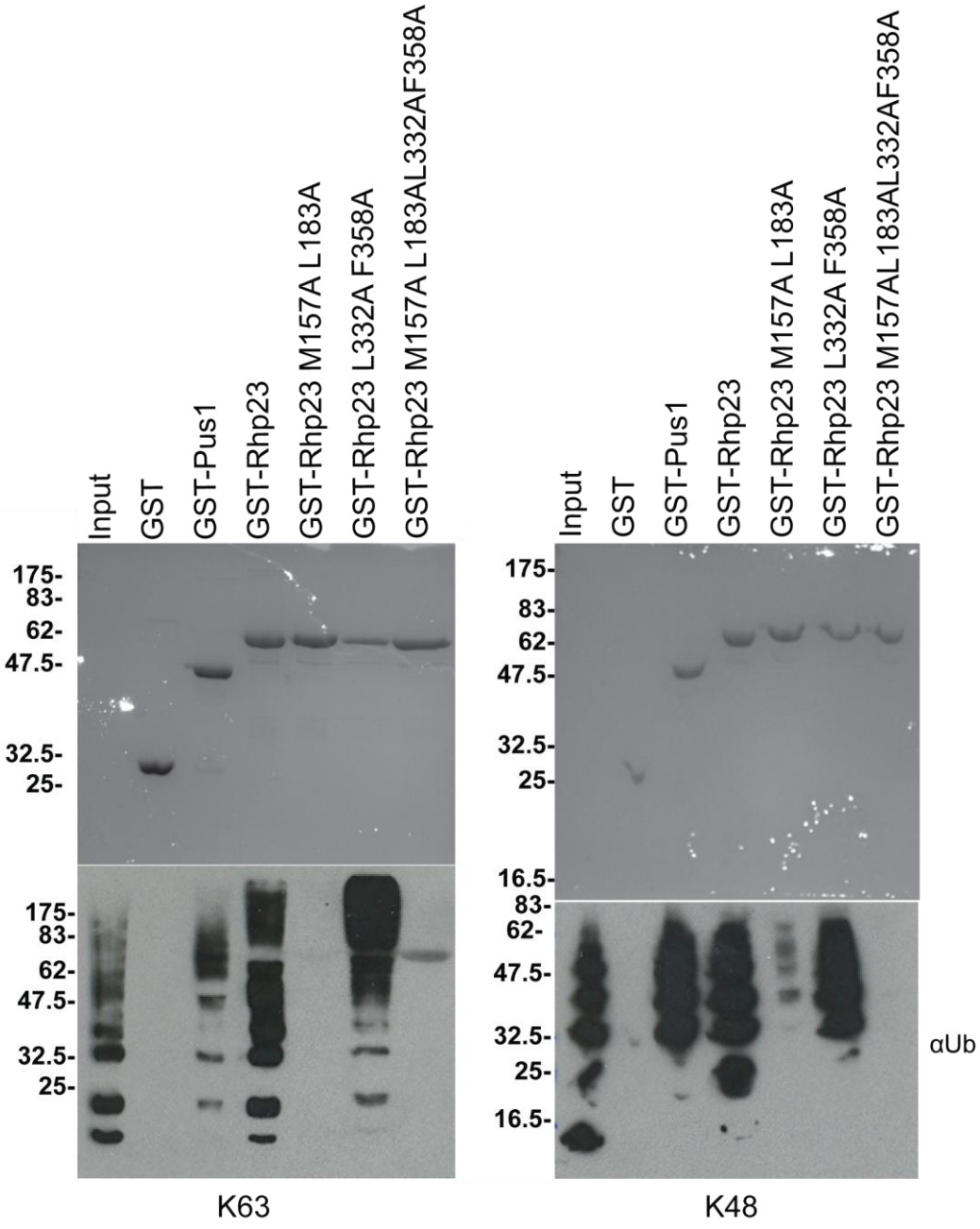


Figure 39: Ubiquitin Binding Assays for Mutated UBA Domains in Full Length Rhp23.
K48 or K63 ubiquitin chains were incubated with GST tagged proteins coupled to beads then washed to remove unbound chains. Proteins were separated on 10% SDS PAGE and either Coomassie stained to show equal loading (top panel) or transferred onto membranes to be probed with anti ubiquitin antibody (bottom panel). The Western blots in the bottom panel show that WT full length protein and the UBA2 mutated protein can bind both types of chains whilst the point mutations in just the UBA1 domain or both domains reduce or inhibit ubiquitin chain binding.

As predicted this experiment demonstrated that when the UBA1 M157A L183A mutations were tested in the full length Rhp23 protein it could no longer bind K63 chains and its ability to bind K48 chains was severely reduced (Figure 39). When both domains were mutated this caused the protein to completely fail to bind either type of chain. Against expectation, the L332A F358A mutation in UBA2 showed no effect on the proteins ability to bind either type of chains. This data suggests that UBA1 is more important in binding ubiquitin chains than UBA2 in the context of the whole protein. This data also implies that the addition of the rest of Rhp23 effects UBA2 function.

6.5 Domain Swap of Pus1 UIM with UBA1 Can Bind Ubiquitin

To further characterise the UBA domains outside of Rhp23 but not in isolation, chimeric constructs were made with Pus1. Amino acids 1-195 of Pus1 were created as a GST fusion constructs, which did not contain the ubiquitin binding UIM domain. This protein was tested for its ability to bind K48 and K63 chains and as seen in Figure 40, no binding was observed. Using homologous PCR the UBA1 or UBA2 domains were grafted onto the end of this construct to create a chimeric protein (Figure 37).

When these proteins were tested for ubiquitin binding, the Pus1ΔUIM+UBA1 construct could bind both chains at a similar level to WT Pus1 and Rhp23 (Figure 40). This showed that the UIM could be replaced with another ubiquitin binding domain. It also demonstrates that the UBA1 domain can be active at the C-terminus and not just as an internal domain. However the Pus1ΔUIM+UBA2 showed little to no chain binding, suggesting that the UBA2 domain does not bind ubiquitin efficiently at the C-terminal of either shuttle protein and may have another role in the context of the protein.

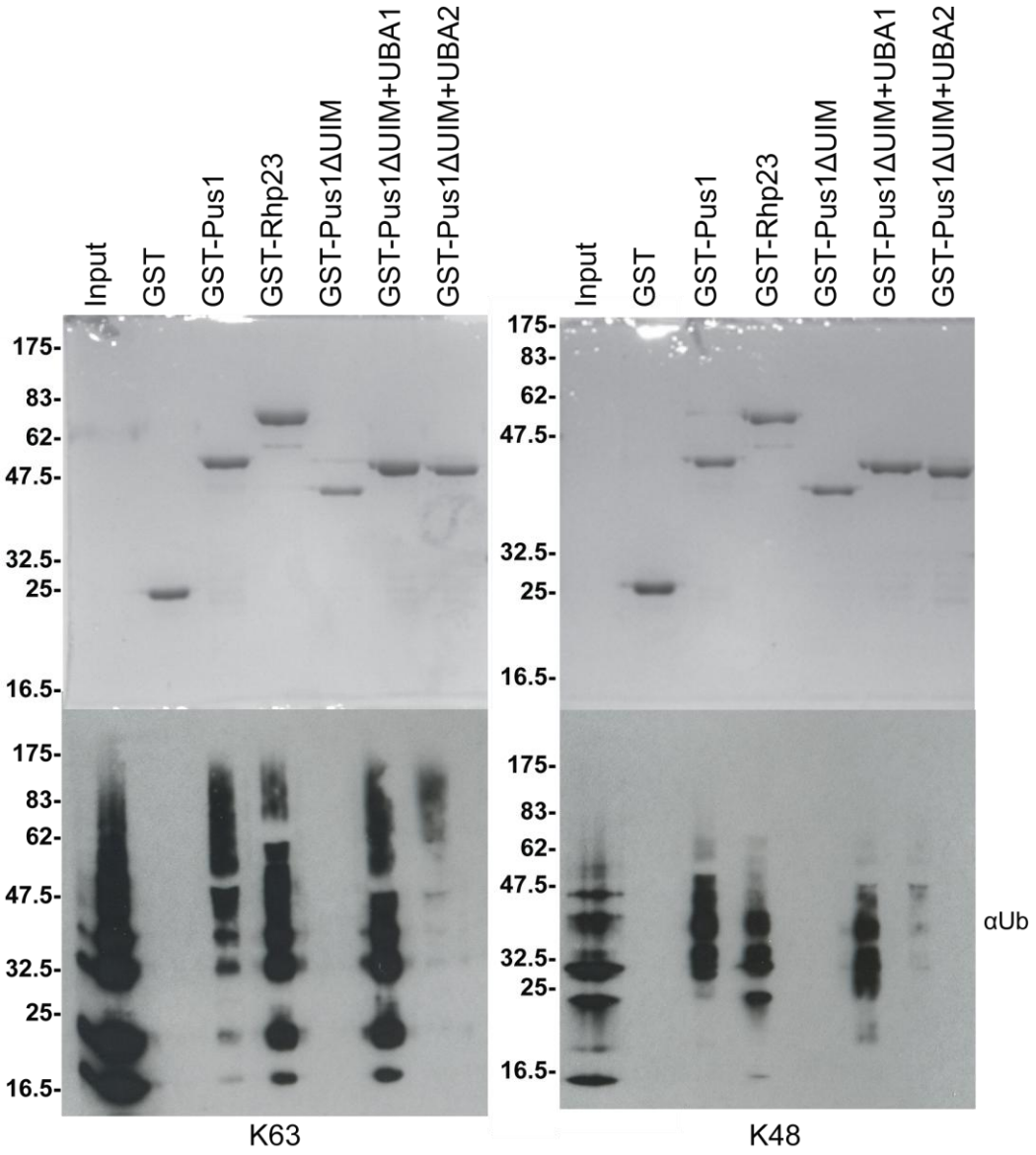


Figure 40: Ubiquitin Binding Assays for UBA Domains Transferred onto Pus1.

GST tagged proteins were coupled to beads and incubated with either K63 or K48 ubiquitin chains. The beads were then washed to remove unbound chains. Proteins were separated on 10% SDS PAGE and either Coomassie stained (top panel) or transferred onto membranes to be probed with anti ubiquitin antibody (bottom panel). The Coomassie stained SDS PAGE gel of input GST fusion protein show equal loading. Western blots within the bottom panel show that WT full length protein could bind both chains but the Pus1 without the UIM could not. The domain swapped Pus1/Rhp23 chimeric proteins show that the UBA1 domain bound both types of chain similar to WT Rhp23 whilst the UBA2 shows little or no chain binding.

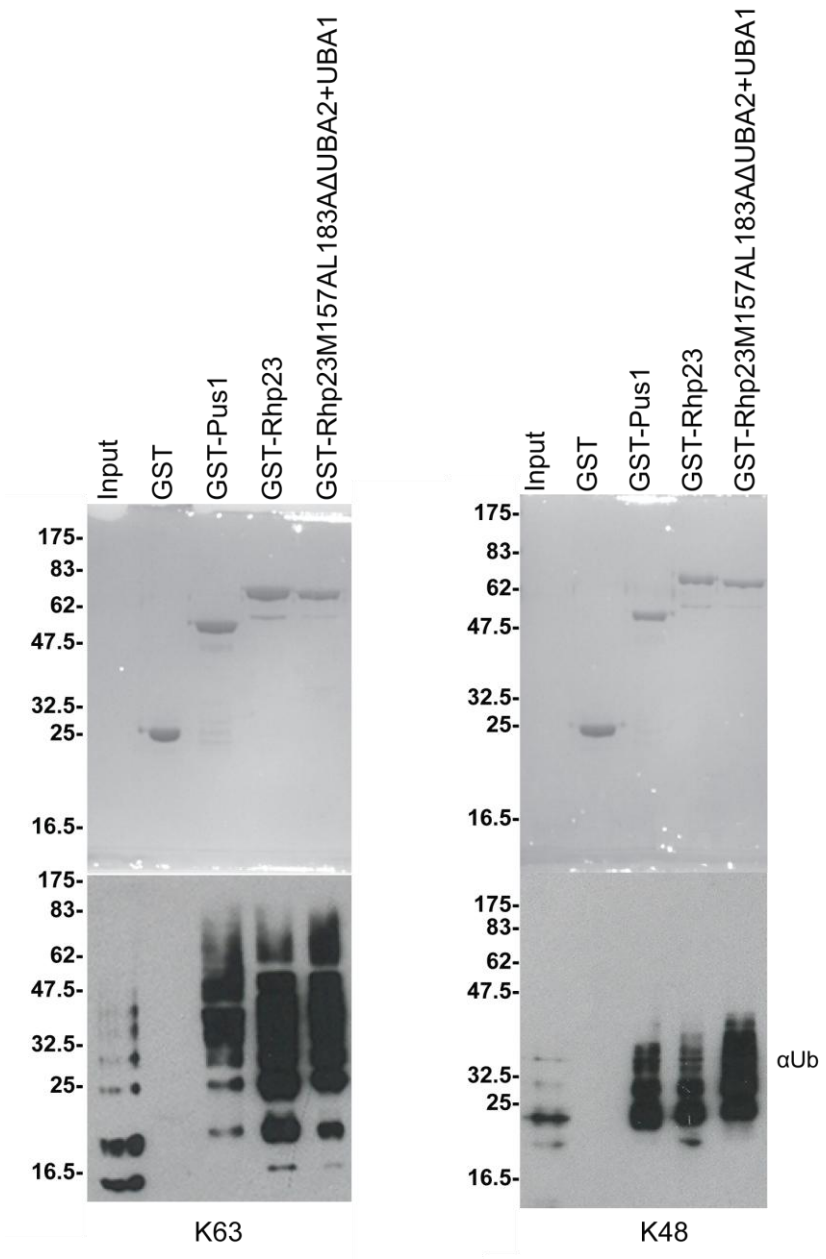


Figure 41: Ubiquitin Binding Assays for Rhp23 M157AL183AΔUBA2+UBA1.

GST tagged proteins were coupled to beads and incubated with either K63 or K48 ubiquitin chains then washed to remove unbound chains. Proteins were the separated on 10% SDS PAGE and either Coomassie stained (top panel) to show equal loading of the input GST fusion proteins or transferred onto membranes to be probed with anti ubiquitin antibody (bottom panel). Western blots show that Rhp23M157AL183AΔUBA2+ UBA1 can bind both K63 and K48 chains at a similar level to WT Rhp23 or better.

6.6 The UBA1 Domain Can Bind Ubiquitin When Placed at the C-terminus

It has been reported that the UBA2 domain may have another function that is not related to ubiquitin binding but instead to protect Rhp23 from becoming a substrate for the proteasome when it is acting as a shuttle factor (Heessen et al. 2005). To test whether it is the context of the domain within the protein, or if it is the domain itself which is important, Rhp23 was constructed with M157A L183A mutations in the internal UBA1 domain. Using the same technique employed to make the Pus1 chimeric proteins, the UBA2 domain was replaced with a non mutated UBA1 domain (Figure 37).

When tested for ubiquitin chain binding, this mutated chimeric protein could bind both chain types equally well, if not better, than WT Rhp23 (Figure 41). Since the mutations in the internal UBA1 abolishes or severely reduced ubiquitin binding, even in the presence of an active UBA2 domain, it can be concluded that the binding seen is due to the UBA1 domain in the UBA2 domain position. This correlates with the Pus1 chimeric protein since both show that the UBA1 domain can carry out its ubiquitin binding function at the C-terminal of a protein which is not its normal protein environment. This result also suggests that there is something unique about the UBA2 domain which causes it to behave in this way.

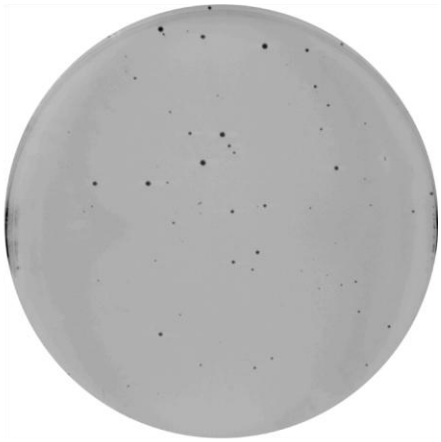
6.7 UBA1 Activity is more Important *in vivo* than UBA2

The function of the UBA domains has to date normally been studied *in vitro* by producing GST tagged minimal domains in isolation to perform binding assays. The study of the individual domains functions *in vivo* has never been performed in *S. pombe*. Previous studies by our lab have shown that the double null mutant *rhp23*Δ*pus1*Δ displays a growth defect at 25^{o}C and that shifting the cells to a restrictive temperature of 36^{o}C causes cell to die. This study also showed that the *rhp23*Δ*pus1*Δ*dph1*Δ mutant was synthetically lethal (Wilkinson et al. 2001).

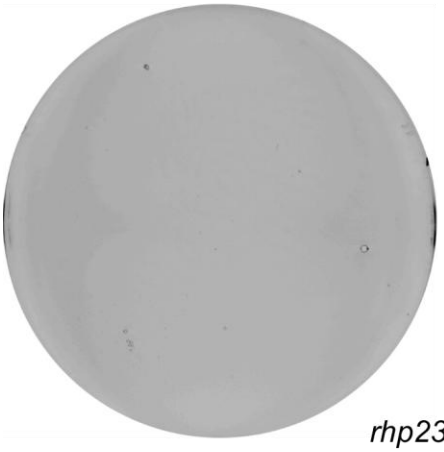
PMG-Leu-Ura-Arg+G418

rhp23Δpus1Δdph1Δ
pREP81

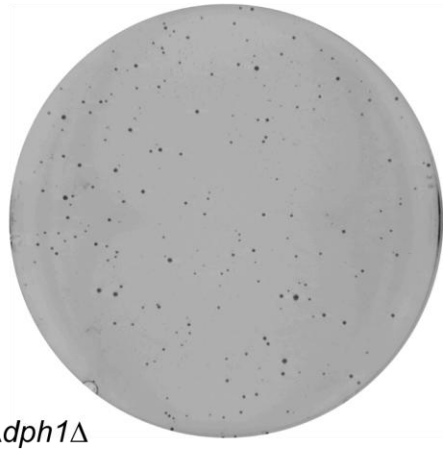
rhp23Δpus1Δdph1Δ
pREP81 Rhp23



rhp23Δpus1Δdph1Δ
pREP81 Rhp23 M157AL183A



rhp23Δpus1Δdph1Δ
pREP81 Rhp23 L332AF358A



rhp23Δpus1Δdph1Δ
pREP81 Rhp23
M157AL183AL332AF358A

Figure 42: Rescue of *pus1*Δ*rhp23*Δ*dph1*Δ Lethal Phenotype Requires an Active UBA1 Domain. *rhp23*Δ*dph1*Δ double mutant was transformed with pREP81 contained WT Rhp23, Rhp23M157AL183A, Rhp23L332F358A, Rhp23M157AL183AL332F358A or an empty vector. These cells were then crossed to *pus1*Δ*dph1*Δ and the asci digested with β-glucuronidase. Spores were plated PMG-Leu-Ura-Arg +G418 to select for *pus1*Δ*rhp23*Δ*dph1*Δ carrying the plasmid and allowed to grow at 25^{o}C. Although a few colonies grew for pREP81, microscopic examination determined that all of these colonies were a result of diploid cells. pRep81 could not rescue the lethality of the triple mutant, neither could the pREP81expressing Rhp23M157AL183A. The pREP81 Rhp23M157AL183AL332F358A gave rise to some colonies, most of which were diploid as seen with empty pREP81, although two colonies appeared to be haploid. Rescue was only observed for pREP81 expressing Rhp23 and Rhp23L332F358A, demonstrating that the UBA1 domain binds ubiquitin chains *in vivo.*

In order to test the function of the two UBA domains *in vivo* pREP81 constructs were made containing the WT and mutant forms of *rhp23* and transformed in *rhp23*Δ *dph1*Δ double mutant. These strains were the crossed to *pus1*Δ*dph1*Δ and the asci digested with β-glucuronidase to perform free spore analysis. The spores were then plated on PMG-Leu-Ura-Arg +G418 to select for *rhp23*Δ*pus1*Δ*dph1*Δ triple mutants carrying the pREP81 plasmid (Figure 42).

This assay revealed that the empty pREP81 plasmid could not rescue the lethality of the triple mutant. Although some colonies grew when expressing empty pREP81, microscopic examination determined that all these colonies contained diploid cells. WT Rhp23 gave rise to colonies formed by haploid cells, indicating that Rhp23 could rescue the *rhp23*Δ*pus1*Δ*dph1*Δ phenotype. Further more, Rhp23 carrying the point mutations in the UBA2 domain were also able to rescue this phenotype, proving the importance of the UBA1 domains role in ubiquitin binding. The corresponding mutations in just the UBA1 domain transformed into the triple mutant resulted in lethality. However, Rhp23 with both UBA domains mutated gave rise to colonies, most of which were due to diploid cells as seen with pREP81, but two colonies appeared to haploid cells. This result was not seen in earlier repeats experiments in which Rhp23 M157AL183AL332AF358A could not rescue the *rhp23*Δ*pus1*Δ*dph1*Δ phenotype. This data correlates with the results of the binding assays and shows for the first time *in vivo* that the UBA1 domain of fission yeast Rhp23 is responsible for recognition and binding of ubiquitin chain and that the UBA2 domain in the context of the whole protein is not enough to allow Rhp23 to perform this process.

6.8 Discussion

In the study of ubiquitin the most common way of investagating into the role of ubiquitin binding domains has been to produce GST tagged minimal domains and assay their ability to bind chains *in vitro*. Recent studies have suggested that this may not be an accurate way of measuring the affinity of domains for different types of chains. There

has been evidence that domains in isolation behave differently than when they are part of a protein. For example, Raasi et al. (2005) showed that hHR23A UBA2 binds K48-Ub_4 chains better than K63-Ub_4 whilst UBA1 prefers K63-Ub_4 to K48-Ub_4. They then went on to show that the addition of the UBL containing N-terminus to UBA1 causes a shift in preference from K63-Ub_4 to K48-Ub_4.

Another major issue was highlighted by Sims et al. (2009), where they demonstrated that GST tagged UBA domains show higher binding affinities for K63 chains due to an artefact of the assay caused by the GST tags forming dimers. When domains were tested for chains binding by NMR comparing GST to non GST tag, the results for the hHR23A UBA1 domain produced a change in K_d values from 4.7µM and 13µM for K63-Ub_4 and K48-Ub_4 respectively to 30µM and 9µM. This was not the only example they found, and they speculate that a vast amount of the data for ubiquitin binding domains may be inaccurate since nearly all studies are carried out using GST tagged protein. They also argue that NMR analysis of untagged protein yields the most accurate reflection of ubiquitin binding.

In light of these finding, further work is needed to accurately determine the functions of UBA domains in their native proteins. Since the data presented here was produced with GST tagged proteins and was not quantitative, Konstantinos Paraskevopoulos from our group and Jonas Boehringer from Jane Endicott's group performed similar studies using fluorescently labelled Ub_2. They tested the same constructs used in this study, but first removed the GST tag then measured the interactions with K48-Ub_2 and K63-Ub_2 using fluorescence anisotropy experiments (Appendix C & Appendix G). The results mirrored what was observed in the binding experiments proving that the interactions were real and not an artefact of the assay.

In order to study the difference in function of isolated and native domains, point mutations were constructed that would inhibit ubiquitin binding but not alter tertiary structure in both isolated domains and full length Rhp23. The GST tagged proteins were then assayed for their ability to bind K48 and K63 chains. The results of the two isolated

UBA domains showed that both were capable of binding to K48 and K63 chains at a lower affinity compared to WT Rhp23 and that the point mutations abolished this binding. This proved that both Rhp23 UBA domains have the ability to recognise ubiquitin chains, as seen in other studies, and in different homologs (Wilkinson et al. 2001; Raasi et al. 2005). It also showed that the mutations predicted to cause a loss of ubiquitin recognition did indeed abolish binding (Ryu et al. 2003).

Unexpectedly, when these assays were repeated with full length proteins, mutations to the UBA1 domain resulted in loss of K63 binding and a dramatic reduction in the ability to bind K48 chains; whereas, corresponding mutations to the UBA2 domain had no visible effect on binding to either chain. The conclusion from these experiments is that the UBA1 domain is responsible for the majority of ubiquitin chain binding, something that would not be predicted from the isolated domain study. This mirrors results produced by Chen & Madura (2002) in *S. cerevisiae* where they used Flag-RAD23 to purify the protein and analyse ubiquitin conjugates bound to WT, mutated and truncated proteins. When they put a single point mutation in the UBA1 domain this caused an 80% reduction in ubiquitin binding whilst a mutation in the UBA2 domain only caused a 20% reduction. However they never showed the effects of these mutations to the domains in isolation or that these mutations had no effect on the protein structure.

The result of this study suggests that the UBA2 domain in isolation behaves differently compared to the full length protein. The difference in results between the isolated domains and the full length protein with mutations suggest that the context of the domain in relation to the protein as a whole may influence its function. This hypothesis has been raised before by Raasi et al. (2005) as they observed a change in chain linkage preference, not a complete loss of ubiquitin binding activity. Therefore to properly understand the UBA domains function they must be studied in the context of the whole protein.

To better dissect the role of the UBA1 and UBA2 domains, their function outside of Rhp23 was tested by forming Pus1ΔUIM chimeric fusion proteins. Pus1 is another

shuttle factor, as well as a subunit of the proteasome, which contains domains of similar function as Rhp23. The removal of the UIM domain rendered the protein unable to bind ubiquitin chains. Therefore, any ubiquitin chain binding affinity generated by grafting a minimal UBA onto this protein would be due to the domain. These chimeric proteins showed that UBA1 again binds both K48 and K63 linked ubiquitin chains with better affinity than UBA2. This is an important result as it shows that the UBA1 domain can bind ubiquitin whether it is internal or at the C-terminus of a protein. However, the UBA2 still cannot recognise chains, even though it is occupying the same protein position as it does in Rhp23. This result, along with the Rhp23 mutation studies, suggests that ubiquitin recognition is not the main function of the UBA2 domain. One explanation for this difference is that the addition of protein to the N-terminus of the UBA2 changes its ability to bind ubiquitin chains. Although they have the same structure, the two domains have very difference sequences. This difference may be the reason why the UBA1 domain's ubiquitin binding function appears to be unaffected by the addition of protein at either end of the domain, yet the addition of protein to the N-terminus of UBA2 dramatically effects its binding ability.

Another interesting observation that arises from this experiment is the UIM domain can be replaced by the UBA1 domain, showing that the main function of both of these domains is to act as receptors for ubiquitin chain binding even though they have a completely different structure. Originally these constructs were cloned into the fission yeast expression vector pREP1 to perform the *in vivo* assay used to test the function of inactive UBA domains. This yielded an unexpected yet very interesting result. When Pus1ΔUIM was transformed into *pus1*Δ a crossed to *rhp23*Δ as a negative control, this protein was able to rescue the growth phenotype *rhp23*Δ*pus1*Δ (Appendix D). When the more stringent assay with *rhp23*Δ*pus1*Δ*dph1*Δ was tested Pus1ΔUIM also rescued the lethal phenotype (data not shown). This protein cannot bind ubiquitin and the only other functional domain present was the vWA domain. The vWA domain interacts with the proteasome and it has been shown that removal of the first 83 amino acids of Rhp23 disrupts this interaction. When N83ΔPus1 and N83ΔPus1ΔUIM constructs were tested

neither could rescue the phenotype. This suggests that the vWA domain plays an important role in the UPS which has yet to be defined, possibly interacting with a yet undiscovered ubiquitin receptor. Since the Pus1ΔUIM could rescue the triple mutant, the chimeric Pus1 proteins could not be tested *in vivo*.

Other functions outside of ubiquitin binding have been proposed for the UBA2 domain. Heessen et al. (2005) presented evidence that the C-terminal position of the UBA2 domain allows it to protect Rhp23 from being degraded by the proteasome when delivering substrates and that removal of the UBA2 domain drastically decreases the half life of Rhp23 *in vivo*. This may explain why UBA2 appears to have no ubiquitin binding ability in the Rhp23 protein. In order to dissect whether it is the position of the UBA2 domain or the domain its self which causes this inactivation, an Rhp23 chimeric protein was created which contained the M157AL183A mutations in the internal UBA1. UBA2 was removed and a WT UBA1 domain put in its place. This Rhp23M157AL183AΔUBA2 +UBA1 protein seemed to bind K48 and K63 chains at a level similar or exceeding WT Rhp23. Since the mutations have been shown to completely abolish ubiquitin binding, this data suggests that the C-terminal UBA1 is responsible for binding. Taken along with the Pus1 chimeric protein results this concludes that it is the UBA1 domain itself which is responsible for its binding efficiency, not its location. This is also consistent with the finding of Heessen et al. (2005) where the UBA2 but not the UBA1 domain could cause stability of UPS substrates when added to the C-terminus. If the UBA2 domain is responsible for protecting Rhp23 from degradation it may be due to a combined result of its sequence and its location, not just its location.

In order to understand the UBA2 role fully Rhp23M157AL183AΔUBA2+UBA1 should be tested for its ability to protect Rhp23 from degradation. This data could explain if any UBA domain at the C-terminal is capable of this function or whether the sequence of this domain plays an important role. Another key experiment would be to swap around the UBA1 and UBA2 domains so that the UBA2 is internal then carry out mutation experiments in a similar manner as previously described in this work. This would allow

further analysis into how the protein environment of a domain affects its function. However, one major problem with this approach would be that swapping the domains is likely to have a severe impact on the protein structure, and it might be unclear how much this would affect ubiquitin binding affinities.

This study is the first time the ubiquitin binding abilities of the two UBA domains have been tested individually in *S. pombe*. Whilst empty pREP81, Rhp23 M157AL183A and Rhp23 M157AL183AL332AF358A could not rescue lethality, Rhp23 and Rhp23 L332AF358A could. The results thereby show that only the UBA1 domain activity is essential for Rhp23s function in the UPS whilst the UBA2 domain appears redundant. This exciting result is consistent with the observations made in the binding assays, but appears to differ with results in budding yeast. A similar experiment concluded that Flag-RAD23 with just a functional UBA1 domain was not enough to rescue the *rad23*Δ*rpn10*Δ phenotype (Chen & Madura 2002). It could be the case that RAD23 is acting different in *S. cerevisiae* compared to Rhp23 in *pombe*. However, *S. pombe* maybe a better *in vivo* assay system for testing ubiquitin binding associated with the UPS since *rad23*Δ*rpn10*Δ does not display any phenotype at permissive temperature and is cold sensitive (*cs*), where even at the lowest temperature tested (13^{o}C) the double mutant showed some growth. This phenotype is not as severe as the *rhp23*Δ*pus1*Δ*dph1*Δ mutant or even the *rhp23*Δ*pus1*Δ mutant in *S. pombe*. Chen & Madura (2002) also tested rescue of *rad23*Δ*rpn10*Δ sensitivity to the arginine analog canavanine. Growth assays showed that neither WT or the mutant Flag-RAD23 could restore the phenotype to WT growth.

From this study it is still unclear what the function of the UBA2 domain is. It has been well established that the UBA domains are not required for Rhp23 role in Nucleotide Excision Repair (NER) after DNA damage (Bertolaet et al. 2001). One observed function of the UBA2 domain is that it protects Rhp23 from being degraded (Heessen et al. 2005). This would fit in with this study as a protection role may not require ubiquitin binding ability of the domain. Rhp23 and its homologs have also been shown to have a

role in preventing degradation of substrates which requires ubiquitin recognition (Ortolan et al. 2000; Clarke et al. 2001; Chen & Madura 2002; Raasi & Pickart 2003).

An attractive hypothesis is that the UBAs compete with proteasomal based receptors and bind chains of the tagged targets, thereby preventing delivery of substrates to the proteasome, and that this action is concentration dependent. Two other observed functions of the ubiquitin binding domains include blocking ubiquitin chain elongation when Rad23 is overexpressed as well as inhibiting deubiquitination of substrates which requires only the minimal ubiquitin binding domains (Ortolan et al. 2000; Hartmann-Petersen, K. B. Hendil & Gordon 2003a). These finding suggest that the binding of the domains may physically block additional or removal of ubiquitin moieties to the chain. Taken together these observations predict that there is an additional layer of regulation provided by the shuttle factors, particularly Rhp23, to control which substrates are degraded and when. However the precise mechanism of how these events occur has yet to be described and it is possible that although the UBA2 domain does not appear to have any ubiquitin binding activity in Rhp23; it may be involved in these processes.

Chapter 7
Discussion

7.1 Role of Kinases Associated Domain

Since its discovery, ubiquitin has been linked to numerous different pathways and its importance as a post translational modification may rival that of phosphorylation. Understanding how ubiquitination is regulated in normal conditions, and the consequences of its mis-regulation, has been of great interest to scientists as it has the potential to lead to new therapies for a multitude of different diseases.

The KA1 domains were originally found in the MARK/Par1/Kin1 family of protein kinases. They are found at the C-terminal of serine/threonine kinases and were originally described as having a conserved aspartic acid residue and ended in a highly conserved ELKL motif. This definition has been widened to include domains which do not end in the ELKL motif and domains which have a conserved glutamic acid residue. Bioinformatics analysis performed by Kay Hofmann (Miltenyi Biotec GmbH, Stoeckhimer) found that three of the known kinases encoded by *S. pombe* contain KA1 domains, Ssp2, Ppk9 and Kin1 whilst another associated domain, which he named the KA2 is found in Chk1.

Previous work has shown that two of these kinases, Ssp2 and Ppk9 can bind to APC/C. This project first set out to determine if the KA1 domain of these proteins was responsible for this interaction, and if so, if this is a function common to all KA domains. The results presented in this work show that all three KA1 and the KA2 domains can bind APC/C. Interestingly, the Cut9-HA strain used to performed *in vitro* binding assays demonstrated that the KA domains exclusively bind APC/C containing unphosphorylated Cut9. This would suggest that there is something specific about this form of APC/C that the KA domains recognise. Yeast two hybrid analysis showed that the KA domains did not interact with any of the core APC/C subunits, but a library screen revealed that the Chk1 KA2 domain could interact with the co-activator Slp1. This interaction was confirmed by binding assays using GST-Slp1, which can interact

with Chk1-HA, and also by native far Westerns for all four KA domains against whole cell extract from a Slp1-GFP strain.

This mode of interaction may explain why the domains appear to select for a certain form of APC/C. Since APC/C is active throughout the cell cycle it utilises two co-activators to recognise specific substrates at different points of the cell cycle. APC/Cs activity is also regulated by phosphorylation which can render it active or inactive depending on the subunits modified and the kinase responsible. A possible explaination could be that Slp1 only binds to APC/C when it contains unphosphorylated Cut9. As a result when the KA domains bind Slp1, they interact with APC/C containing unphosphorylated Cut9. It remains undetermined from these results whether the KA domains can bind free Slp1 or only when it is part of the APC/C complex. However, in the *in vitro* binding assay GST-Slp1 could only bind Chk1-HA and not Ssp2-HA or Ppk9-HA, yet in the native far Westerns the GST-KA domains could bind Slp1-GFP. This may suggest that the domains only interact with Slp1 when it is part of APC/C^{Slp1}.

The second aim of this project was to determine if these kinases have any role in regulating APC/C. Phosphorylation assays using GST tagged full length Ssp2, Ppk9 and Kin1 and TAP tagged purified APC/C showed that these kinases phosphorylated a specific ~30kDa protein. None of the core APC/C subunits or its co-activators correlate to this molecular weight. However Sczaniecka et al. (2008) performed similar purifications of APC/C and showed that the MCC subunit Mad2 co-purified with APC/C and appear in Western analysis at ~ 30kDa, making it a candidate. Repeat phosphorylation assay with GST-Mad2 showed that Ssp2 and Ppk9 can phosphorylate Mad2. Studies have shown that the phosphorylation state of Mad2 regulates its ability to bind Slp1 and form the MCC which inhibits APC/C^{Slp1} activity. However the kinases responsible for phosphorylating Slp1 have yet to be identified. Therefore these are the first kinases reported that can act on Mad2.

Another interesting result seen from the phosphorylation assays was that full length proteins could not autophosphorylate, but ΔKAs could. This would support the

hypothesis that the KA domain may act as an auto-inhibitor domain for the N-terminal kinase domain. This is also supported by the tertiary structure of Ssp2 and Ppk9. Together this has lead to the proposed model in Figure 19, in which the KA domains bind to and inhibit the kinase domains. After a cellular event the KA domains then bind to Slp1 in the APC/C complex. The kinase domain is then free to act on Mad2 which also binds Slp1 as part of the MCC. This phosphorylation causes the MCC to dissociate and leaves APC/C^{Slp1} free to act.

These findings have given rise to other questions not addressed in this work. It would be important to study how the addition of the KA containing kinases affects APC/C^{Slp1} activity in a cell free system. The exact binding site of the domains on Slp1 is not known, or if KA1 and KA2 recognise the same site which could be determined by competition studies. Also, the phosphorylation sites on Mad2 need to be identified to see if all four kinases can phosphorylate the same residues.

All four null mutants of the KA containing kinases are viable, as are every double, triple and even the quadruple mutant. Genetic analysis performed in this study could not find a phenotype that was common to all four mutants. However *kin1*Δ was linked to the UPS since a double mutant with the proteasome mutant *mts3.1* was synthetically lethal. It was also the only mutant which showed an increase in mis-segregation of chromosomes, both of which is consistent with Kin1 regulating APC/C activity.

7.2 Role of Ubiquitin Binding Domains in Rhp23

The second project presented here was concerned with dissecting the role of the two UBA domains of Rhp23 in *S. pombe*. Unlike the shuttle factors Pus1 and Dph1, Rhp23 has two UBD; an internal UBA1 and a C-terminal UBA2. These minimal domains were expressed as GST fusion proteins and assayed for K48 and K63 ubiquitin chain binding ability. Both of these domains can bind both chain types but two point mutants in either domain, which were predicted not to alter the structure, abolished ubiquitin binding. However, when these point mutations were tested in full length Rhp23, the binding

assay revealed that the UBA1 domain of Rhp23 seemed to be responsible for the majority of Rhp23 ability to bind K48 and K63 chains. Mutating the UBA2 domain has little effect on ubiquitin binding.

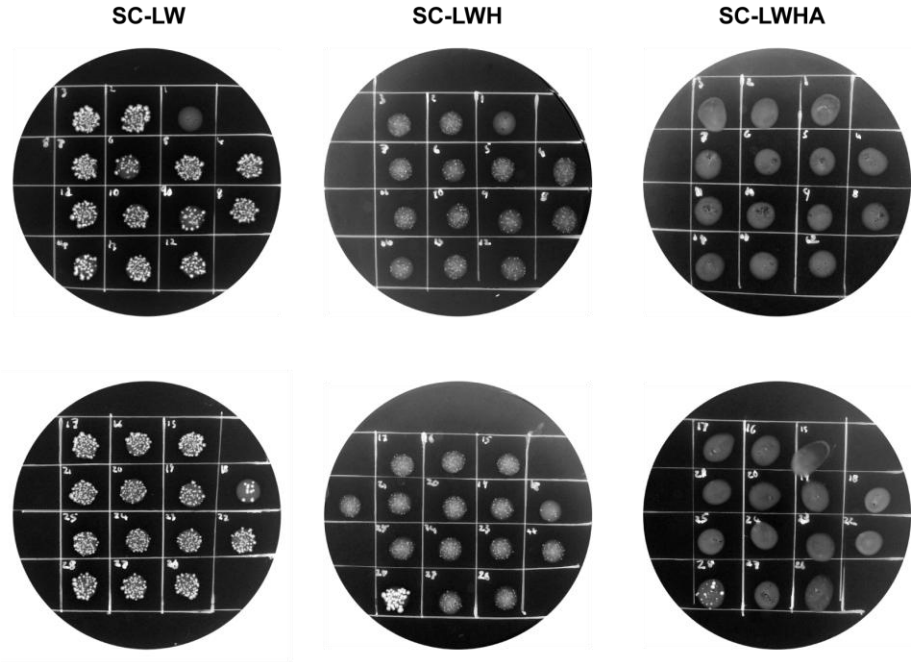
To further test the two domains ubiquitin binding activity chimeric proteins were constructed. The shuttle protein Pus1 with its UIM domain removed was used since it could not bind ubiquitin. The minimal UBA domains were then grafted onto this protein. Binding assays showed that Pus1ΔUIMUBA1 could bind both types of ubiquitin chains, but Pus1ΔUIMUBA2 could not. A further chimeric protein was made in which Rhp23 had its UBA1 domain mutated and its UBA2 domain replaced with a WT UBA1 domain. This Rhp23M157AL183AΔUBA2+UBA1 protein could bind K48 and K63 chains at a similar level to WT Rhp23. These experiments would suggest that the UBA1 domain can bind ubiquitin regardless of whether it is an isolated domain or as part of a protein, either as an internal or C-terminal domain. However, the UBA2 domain only appears to bind ubiquitin when it is an isolated minimal domain. The mutation experiments and chimeric proteins show that the domain has little binding activity when part of a protein.

These results are supported by quantitative binding assays performed in collaboration with Jane Endicott's group (Department of Biochemistry, University of Oxford). The same WT and mutated Rhp23 proteins were expressed and the GST tag cleaved off. These proteins were subjected to fluorescence anisotropy experiments to measure binding constants which mirrored what was seen by Western blot analysis.

in vivo analysis of the Rhp23 UBA domains has never been performed in *S. pombe*. *rhp23*Δ*pus1*Δ*dph1*Δ strains were created that expressed pREP containing WT or mutated Rhp23 to look for rescue. These results showed that WT and UBA2 mutated Rhp23 could rescue the triple mutant, were as the UBA1 mutated Rhp23and UBA1 and 2 mutated Rhp23 could not. Taken together, these results show that UBA1 domain is more important than the UBA2 domain in the UPS. The Western blot analysis show that UBA domains can behave differently in isolation compared to in a full length protein,

suggesting that the protein environment can alter the domains function, and the only accurate way to determine a role of the domain is to study it as part of a protein. Although this work does not determine a role for the UBA2 domain, Heessen et al. (2005) demonstrate that the UBA2 domain is needed to protect Rhp23 from being degraded by the proteasome. Together this data suggests that the UBA1 domain is responsible for binding ubiquitin, regardless of its position in the protein, whilst the UBA2 domain may be responsible for protecting the protein from becoming a proteasome substrate itself. Although Heessen et al. (2005) presented some evidence to suggest that this function is specific to UBA2 domain; it would be interesting to repeat their assay using the Rhp23M157AL183AΔUBA2+UBA1 construct to determine whether the UBA1 domain can also perform this function when it occupies the UBA2 position.

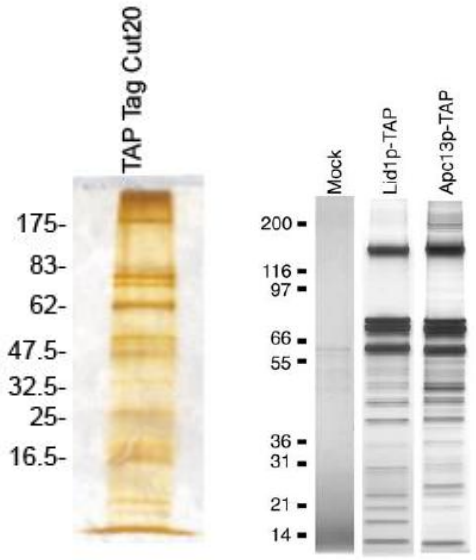
Chapter 8
Appendix



No.	Bait	Prey	No.	Bait	Prey
1	water	water	15	*nuc2*	*chk1 KA2*
2	empty	empty	16	*apc5*	*ssp2 KA1*
3	empty	*apc13*	17	*apc5*	*ppk9 KA1*
4	*nuc2*	empty	18	*apc5*	*kin1 KA1*
5	*apc5*	empty	19	*apc5*	*chk1 KA2*
6	empty	*cut20*	20	*ssp2 KA1*	*cut20*
7	empty	*cut23*	21	*ppk9 KA1*	*cut20*
8	*ssp2 KA1*	*apc13*	22	*kin1 KA1*	*cut20*
9	*ppk9 KA1*	*apc13*	23	*chk1 KA2*	*cut20*
10	*kin1 KA1*	*apc13*	24	*ssp2 KA1*	*cut23*
11	*chk1 KA2*	*apc13*	25	*ppk9 KA1*	*cut23*
12	*nuc2*	*ssp2 KA1*	26	*kin1 KA1*	*cut23*
13	*nuc2*	*ppk9 KA1*	27	*chk1 KA2*	*cut23*
14	*nuc2*	*kin1 KA1*	28	*Snf1*	*Snf4*

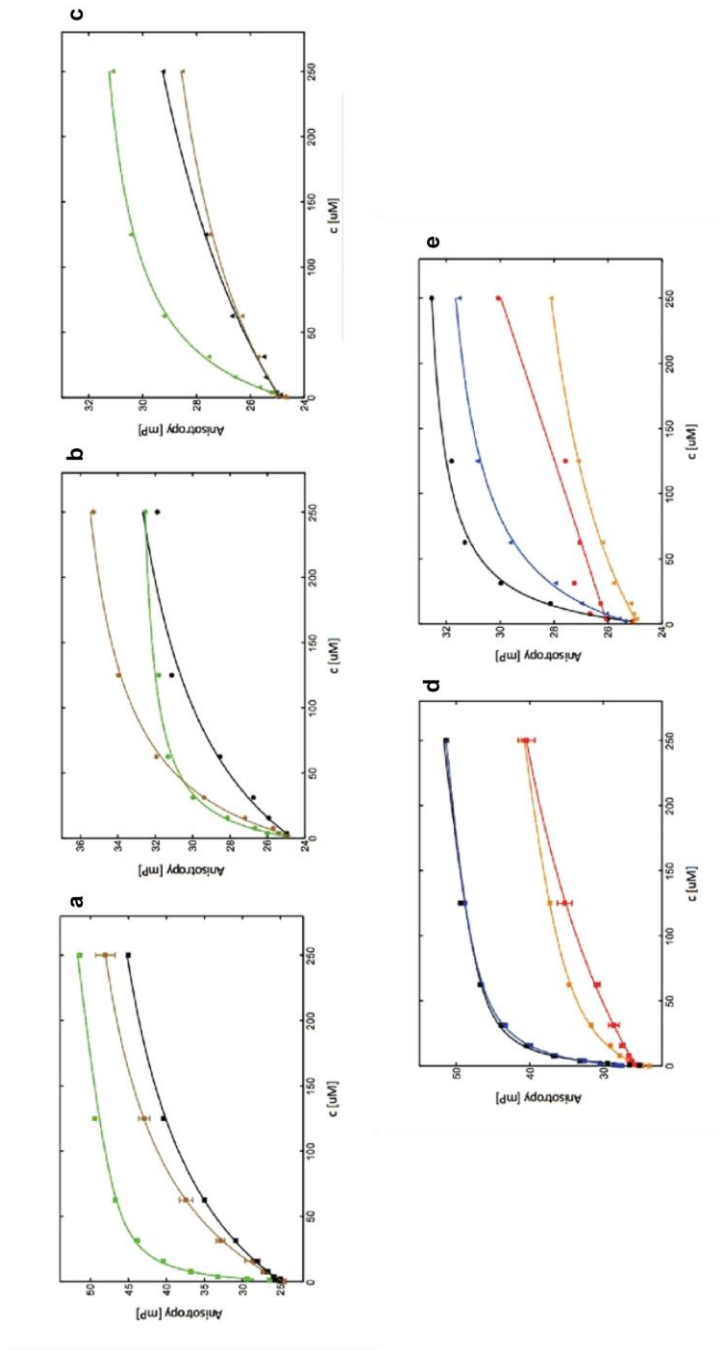
Appendix A: Example of Yeast Two Hydrid Assay.

The J69-4A budding yeast strain was transformed with bait and prey constructs indicated in the table and spotted directly onto plates. SC-LW media was used to test for successful transformation, SC-LWH to look for weak interactions and SC-LWHA to test for strong interactions. Each construct was tested with the opposite empty vector to rule out self activation. Snf1 and Snf4 were used as a positive control whilst the two empty vectors were used as negative controls.



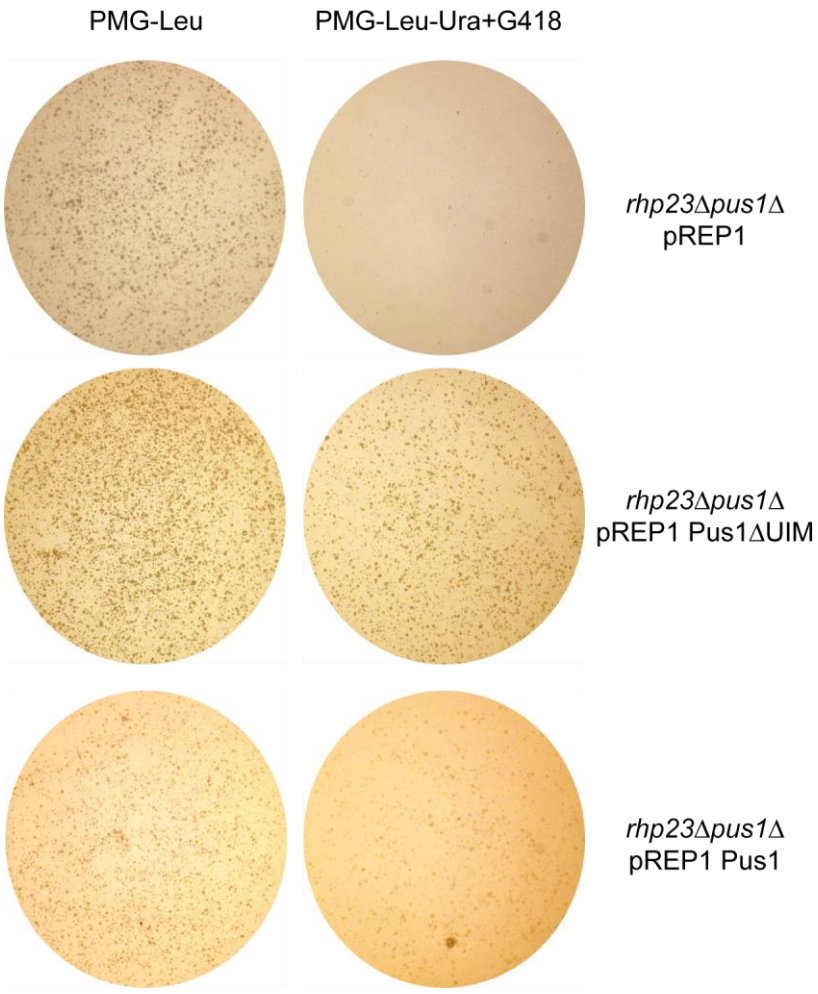
Appendix B: TAP Tag Purification of APC/C.

TAP tag purification of APC/C from Cut20/Lid1 strain which was separated on SDS PAGE gel then silver stained. Banding pattern was comparable to similar staining performed by Yoon et al. (2002) on purified APC/C from the same TAP tag lid1 strain and Tap tag Apc13 strain



Appendix C: Binding Affinities for Full Length Rhp23 and UBA Domains.

a) Full length Rhp23 binding to K48-Ub_2 (green) K63-Ub_2 (black) mono-Ub (brown). **b)** UBA1 domain binding to K48-Ub_2 K63-Ub_2 mono-Ub. **c)** UBA2 domain binding to K48-Ub_2 K63-Ub_2 mono-Ub. d) Binding of K48-Ub_2 to WT Rhp23 (black), Rhp23 M157AL183A (orange) Rhp23 L332AF358A (blue) Rhp23 M157AL183AL332AF358A (red). **e)** Binding UBA1 (black) UBA2 (blue) UBA1 M157AL183A (red) UBA2 L332AF358A (orange)



Appendix D: *in vivo* Assay of Pus1ΔUIM Rescue of *pus1*Δ::ura*rhp23*Δ::G418.

*pus1*Δ::ura transformed with pREP1 constructs and crossed to *rhp23*Δ::G418. Free spore analysis was performed and spores plated onto PMG-Leu to show the presence of spore carrying the plasmid, or PMG-Leu-Ura+G418 to select for *pus1*Δ::ura *rhp23*Δ::G418 double mutants carrying the plasmid. Empty pREP1 could not rescue the double mutants, unlike the Pus1 control. However, Pus1ΔUIM could rescue the phenotype even though this protein is unable to bind ubiquitin chains.

Name	Forward	Reverse
pENTR/D TOPO Ssp2	5'-CACCATGCAACCGCAGGAGGTTGAT	5'-TCATGCAGAAAAATAACTTGC
pENTR/D TOPO Ssp2 (446) KA	5'-CACCAAGAGTCGCCGTAACAAATGGC	5'-TCATGCAGAAAAATAACTTGC
pDONR 221 Ssp2 Δ KA	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTCGATGCAACCGCAGGAGGTTGA	5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCTCATTTGCGAGTGGATTG
pENTR/D TOPO Ppk9	5'-CACCATGCAAACCCCTTCTTCTGCA	5'-TTATGCAATTTTAAGGATCAA
pENTR/D TOPO Ppk9 (421) KA	5'-CACCCGACTTCGTTCTTTACGCTG	5'-TTATGCAATTTTAAGGATCAA
pDONR 221 Ppk9 Δ KA	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTCGATGCAAACCCCTTCTTCTGC	5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCTTAGTTGGGGGCGGGAGGTCGGG
pENTR/D TOPO Kin1 (751) KA	5'-CACCAAGCCTGTTTTCCTTAAAGG	5'-TCACAATTTTAATTCGTTAAG
pDONR 221 Kin1 Δ KA	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTCGATGGAGTACCGTACCAATAA	5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCTCATATAGGATCCAATTTATCCG
pENTR/D TOPO Chk1 (393) KA	5'-CACCGAAAGACTCACTAGGTTTTATTCT	5'-TTAATTTTGTGAAACATCTGT
pDONR 221 Chk1 Δ KA	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTCGATGGCTCAAAAATTAGATAA	5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCTTAAGGAGGACAAATTTCG
M13	5'-GTAAAACGACGGCCAG	5'-CAGGAAACAGCTATGAC
pGBT9	5'-TCATCGGAAGAGAGTAG	5'-AATCATAAGAAATTCGCCC
pGAD424	5'-TACCACTACAATGGATG	5'-TTTCAGTATCTACGATTCATAG
pGEX	5'- GGGCTGGCAAGCCACGTTTGGTG	5'- CCGGAGCTGCATGTGTCAGCGG
pREP1	5' CAATCTCATTCTCACTTTCTG	5'- CGTAATATGCAGCTTGAATG
FA6NAT	5'-GAATTCGAGCTCGTTTAAAC	5'-CGGATCCCCGGGTTAATTAA
Rhp23 M157A	5'-GTCGAAATATGGTAGAAGCGGATACGAACGCAGCG	5'-CGCTGCGTTCGTATCCCGCTTCTACCATATTTTCGAC
Rhp23 L183A	5'-GGCAGTGGAATACTTAGCAACTGGTATTCCCGAAG	5'-CTTCGGGAATAGTTGCTAAGTATTCCACTGCC
Rhp23 L332A	5'-TAGATTATGTCAAGCTGGCTTCGACAGAAA	5'-TTTCTGTCGAAGCCAGCTTGACATAATCTA
Rhp23 F358A	5'-GCTGCTAATACCTTGCCGAGCATGGACATG	5'-CATGTCCATGCTCGGCAAGGTAATTAGCAGC
Pus1ΔUIM for UBA1 Domain	5'- ACGCGTCGACATGGTGTTAGAAGCAACGATGA	5'-TCGACAGCAACATTTCGTTG TTGAGAAGCAACTACACCTTGTCCAA
Pus1ΔUIM for UBA2 Domain	5'- ACGCGTCGACATGGTGTTAGAAGCAACGATGA	5'-CAGATTCTTCTTGAGTAATTTGAAT TTGAGAAGCAACTACACCTTGTCCAA
UBA1 for Pus1 ΔUIM	5'-TTGGACAAGGTGTAGTTGCTTCTCAA CAACGAAATGTTGCTGTCGA	5'-CGGGATCCTCA AATGTCTTCGGGAATACC
UBA2 for Pus1 ΔUIM	5'-TTGGACAAGGTGTAGTTGCTTCTCAA ATTCAAATTACTCAAGAAGAATCTG	5'-CGGGATCCTTAAGGTTCATCCTCAGATTCATGTCC
pREP1_Rhp23	5'-ACGCGTCGACGTATGAATTTGACATTCAAAAATCTACAGCAG	5'-CGCGGATCCGCTTAAGGTTCATCCTCAGATTCATGT
pREP1_Pus1	5'- ACGCGTCGACATGGTGTTAGAAGCAACGATGA	5'-CGCGGATCCTCATTCTTGCATTTTTTTATCC
pREP1_pus1ΔN83	5'-ACGCGTCGACAACGCAAAGTTTGGCGATGG	5'-CGCGGATCCTCATTCTTGCATTTTTTTATCC
pREP1_pus1ΔN83ΔUIM	5'-ACGCGTCGACAACGCAAAGTTTGGCGATGG	5'-CGCGGATCCTTATTGAGAAGCAACTACACCTTG
pREP1_pus1ΔUIM	5'-ACGCGTCGACAACGCAAAGTTTGGCGATGG	5'-CGCGGATCCTTATTGAGAAGCAACTACACCTTG
	5'- ACGCGTCGACATGGTGTTAGAAGCAACGATGA	5'-CGCGGATCCTTATTGAGAAGCAACTACACCTTG

Name	Forward	Reverse
pREP1_pus1ΔUIMUBA1	5'-ACGCGTCGACATGGTGTTAGAAGCAACGATGA	5'-CGGGATCCTCAAATGTCTTCGGGAATACC
pREP1_pus1ΔUIMUBA2	5'-ACGCGTCGACATGGTGTTAGAAGCAACGATGA	5'-CGGGATCCTTAAGGTTCATCCTCAGATTCATGTCC
pGEX6P1_Rhp23	5'-CGCGGATCCATGAATTTGACATTCAAAAATCTAC	5'-ACGCGTCGACGTTAAGGTTCATCCTCAGATTCATGTCC
pGEX6P1_pus1	5'-CGCGGATCCATGGTGTTAGAAGCAACGATGATTC	5'-ACGCGTCGACGTCATTCTTGCATTTTTTTATCC
pGEX6P1_pus1ΔN83	5'-CGCGGATCCAACGCAAAGTTTGGCGATGGT	5'-ACGCGTCGACGTCATTCTTGCATTTTTTTATCC
pGEX6P1_pus1ΔN83ΔUIM	5'-CGCGGATCCAACGCAAAGTTTGGCGATGGT	5'-ACGCGTCGACGTTATTGAGAAGCAACTACACCTTGTCCA
pGEX6P1_pus1 ΔUIM	5'-CGCGGATCCATGGTGTTAGAAGCAACGATGATTC	5'-ACGCGTCGACGTTAAATGTCTTCGGGAATACCAGTTAAT
pGEX6P1_pus1 ΔUIMUBA1	5'-CGCGGATCCATGGTGTTAGAAGCAACGATGATTC	5'ACGCGTCGACGTTAAGGTTCATCCTCAGATCATGTCC
pGEX6P1_pus1 ΔUIMUBA2	5'-CGCGGATCCCAACGAAATGTTGCTGTCGAAAATA	5'-ACGCGTCGACGTTAAATGTCTTCGGGAATACCAGTTAAT
pGEX6P1_UBA1	5'-CGCGGATCCCATTCAAATTACTCAAGAAGAATCTG	5'-ACGCGTCGACGTTAAGGTTCATCCTCAGATTCATGTCC
pGEX6P1_UBA2	'-ATCATGGCATGGATGATCTTAAGTCATCTTTCCCATTTTTAGATTTGTGTGCTAT-	5'-TAGATAAATGGGAAATTTCAGTTATCAGAGAAAACCCGCATGAAAAGTAA
ssp2-HA	GCTGGTGTGCAAGTTATTTTCTGCACGGATCCCCGGGTTAATTAA	AAGGACTCAATAAACTCCTAACATTCAGTCGAATTCGAGCTCGTTTAAAC
ppk9-HA	5'-ATGATTCTATAAAGTACACTTCATCTTACAGTCCAATGCCATTTTTAGAAGTTGT	5'-TTCCGTACTAGGAATTACTGTGAGTATGGACAAACATTCACATTTAATGCA
	CAAACAATTGATCCTTAAAATTGCACGGATCCCCGGGTTAATTAA	TATACACTACTTCCACTCTTTAACTTCATGGAATTCGAGCTCGTTTAAAC
kin1-HA	5'-GCGTTTCTTTTCATCGAATTAGTGGAAACTCATGGCAATATAAGACATTGGCTA	5'-TTTTTTGGTATTTTATAGTTATGGGTTAATAATAATATTACTGCTAGATAAGTA
	GTCGTATTCTTAACGAATTAA AATTGCGGATCCCCGGGTTAATTA A	TCAACAAAAAACGTCGTATTAGACTATGAATTCGAGCTCGTTTAAAC
pGEX6p1_mad2	5'-CCGGAATTCATGTCTAGCGTTCCCATAAGAA	5'-ACGCGTCGACGTCTAAGGATTCACTCGATATGCA
pGBT9_ssp2ΔKA	5'-CCGGAATTCAAGAGTCGCCGTAAC	5'-CCGACGTCGACTCACAATTTAATTCGTT
pGBT9_ppk9ΔKA	5'-CCGGAATTCCGACTTCGTTCTTTAC	5'-CCGACGTCGACTTATGCAATTTTAAGGA
pGBT9_kin1ΔKA	5'-CCGGAATTCAAGCCTGTTTTCCTTAAA	5'-CCGACGTCGACTCACAATTTAATTCGTT
pGBT9_chk1ΔKA	5'-CCGGAATTCGAAAGACTCACTAG	5'-CCGACGTCGACTTAATTTTGTGAAACA
pGBT9_slp1	5'-CGCGGATCCGCATGGAGATAGCAGGTAATTC	5'-CGCGGATCCTCAACGGATTGTTATGCTGC
pGBT9_rev7	5'-CCGGAATTCATGGAAAACGACACAGGATG	5'-ACGCGTCGACCTAATCCGAATTTGTATATT
pGBT9_ste9	5'-CGCGGATCCGCATGGATGAATTTGATGGGTT	5'-CGCGGATCCATGGATGAATTTGATGGGTT
pGBT9_mad2	5'-CCGGAATTCATGTCTAGCGTTCCCATAAG	5'-ACGCGTCGACGCTAAGGATTCACTCGATATGCA
pGBT9_cut4	5'-CGCGGATCCGCATGCTAGAATTAAAAACAAATGTTATTCAA	5'-CGCGGATCCTTATACAGATTCTTCATTATGATTATG
pGBT9_cut9	5'-CCGGAATTCATGGTCGTTAAACGAACGCAAACTGACTCT	5'-ACGCGTCGACTTATCGTTGCTCTGAGACATTACCTT
pGBT9_cut20	5'-CGCGGATCCGCATGGTGTCAAAATCTTTCA	5'-ACGCGTCGACTTAAAAAGAGAATAAACGAT
pGBT9_cut23	5'-ACGCGTCGACGTATGACTGTGTCGCTGAATA	5'-ACGCGTCGACTTAATCATATGAATGCTCCATC
pGBT9_APC2	5'-ACGCGTCGACGTATGAATGATACTGATCTGT	5'-ACGCGTCGACTTATTTGAGCTTATAAGCTC
pGBT9_APC5	5'-CGCGGATCCGCATGGTAACACCAATATTTC	5'-CGCGGATCCTTAGTTTAGCAAATTGTCGG
pGBT9_APC10	5'-CCGGAATTCATGGCGCAAATTCGACAAGA	5'-ACGCGTCGACTTACCGTAATTGGTTTCTACT
pGBT9_APC11	5'-CCGGAATTCATGAAGGTGAAAATACTGAG	5'-ACGCGTCGACTTATTGTGTTTCAGACTTTT
pGBT9_APC13	5'-CCGGAATTCATGGATTCGAATTACAATT	5'-ACGCGTCGACTCATCTAAAATACCGCATAC
pGBT9_APC14	5'-CCGGAATTCATGGATCCGTTTGCTGGTAC	5'-ACGCGTCGACTCACCATTCAGCCAACTGGT
pGBT9_APC15	5'-CCGGAATTCATGTCGTTTATGTCGTTGAT	5'-CGCGGATCCTTAATTTTGAAATCCGTATT
pGBT9_Nuc2	5'-CCGGAATTCATGACAGATCGATTGAAATG	5'-ACGCGTCGACTAAGTTTCCAGATTCCTATA
pGBT9_Hcn1	5'-CCGGAATTCATGTTACGAAGAAATCCTA	5'-ACGCGTCGACCTATGATTGAGCGGCTCCAC

Appendix E: Primers Used During This Work.

Time (hours)	WT			*ssp2*Δ			*ppk9*Δ			*kin1*Δ			*chk1*Δ		
0	0.32	0.49	0.32	0.24	0.42	0.18	0.31	0.31	0.36	0.28	0.48	0.50	0.34	0.54	0.35
1	0.39	0.60	0.38	0.30	0.46	0.24	0.34	0.35	0.39	0.31	0.55	0.52	0.38	0.57	0.46
2	0.51	0.69	0.44	0.37	0.54	0.28	0.40	0.46	0.47	0.36	0.64	0.62	0.49	0.68	0.56
3	0.56	0.77	0.51	0.43	0.64	0.34	0.47	0.53	0.54	0.45	0.72	0.69	0.57	0.81	0.68
4	0.66	0.88	0.61	0.49	0.72	0.39	0.53	0.60	0.64	0.51	0.80	0.80	0.70	0.92	0.79
5	0.74	0.97	0.71	0.58	0.83	0.48	0.64	0.74	0.74	0.58	0.94	0.89	0.82	1.08	0.93
6	0.86	1.14	0.81	0.66	0.96	0.57	0.77	0.84	0.85	0.68	1.06	1.03	0.97	1.23	1.08
7	0.97	1.23	0.91	0.76	1.10	0.63	0.84	0.94	0.97	0.77	1.21	1.18	1.10	1.39	1.21
8	1.08	1.36	1.03	0.85	1.26	0.74	0.97	1.09	1.09	0.87	1.32	1.35	1.29	1.48	1.34

Appendix F: Time Course of O.D from WT and KA Kinase Strains.

YES was inoculated with WT *ssp2*Δ, *ppk9*Δ, *kin1*Δ or *chk1*Δ and grown over night at 25^{o}C. The O.D.$_{600}$ was taken every hour for 8 hours. Three repeats were performed for each strain.

Construct	K48-Ub_2	K63-Ub_2	Mono Ub
Rhp23 WT	7.0 μM	103μM	138μM
Rhp23 M157AL183A	31μM	-	-
Rhp23 L332AF358A	10.9 μM	-	-
Rhp23 M157AL183A L332AF358A	>200μM	-	-
UBA1	19μM	51μM	126μM
UBA1 M157AL183A		-	-
UBA2	48μM	>200μM	>200μM
UBA2 L332AF358A	>200 μM	-	-

Appendix G: Binding Constants for Full Length Rhp23 and Isolated UBA Domains.

K_d values were calculated full length and isolated domain proteins without the GST tagged by fluorescence anisotropy experiments performed by Konstantinos Paraskevopoulos and Jonas Boehringer.

Chapter 9
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